Respiratory Medicine

Series Editors: Sharon I. S. Rounds · Anne Dixon · Lynn M. Schnapp

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The Microbiome in Respiratory Disease

Principles, Tools and Applications

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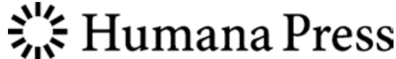
Editors

The Microbiome in Respiratory Disease

Principles, Tools and Applications



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Preface

The human microbiome plays a crucial role in health and disease, which has captured the attention and fascination of many scientists, clinicians, and the interested public. Research on the topic has grown exponentially, but curiously less attention was paid early on to the potential influence of the microbiome in respiratory diseases. This has changed dramatically and rapidly in recent years. We now have a solid foundation of knowledge coupled with a continually growing number of studies, which together are elucidating the impact and processes by which microbiota can shape a variety of respiratory diseases and their clinical outcomes.

Like many things in life and in academics, this book grew from serendipitous discussions and opportunity. When initially asked to consider developing this volume for the American Thoracic Society Respiratory Medicine book series, I viewed the inquiry as (a) evidence of wider recognition about the importance of understanding the microbiome's impact on respiratory disease, and (b) an opportunity to channel smaller-scale efforts that were ongoing at the time and create a larger resource for interested readers. The timing was also fortuitous, given crucial knowledge advances over the last decade. While the gut microbiome in general has garnered the most attention given its outsized microbial biomass, the field has blossomed with a now enriched understanding of the respiratory microbiome. Evidence has emerged that the distal lower airways are not wholly sterile, contrary to historical teaching. Furthermore, upper and lower airway microbiota are quite different, which has broad and potentially important implications for how they influence diseases affecting respective niches in the respiratory system.

The purpose of this book is two-fold and organized as such. The first part (I) aims to provide a foundation in investigative approaches to the microbiome, from study design to technical considerations and computational analyses. Key issues and potential pitfalls that are somewhat unique to studying the respiratory microbiome are highlighted. The second part (II) presents application of such approaches to the study of specific respiratory diseases. We have included discussion of the upper airways/sinuses and also chronic and acute disease conditions. The chapters in this part aim to synthesize evidence from best-practice studies examining the associations or potential mechanisms by which microbiota influence respiratory disease.

These days, research is more and more interdisciplinary, which is invaluable and welcome, particularly in the microbiome field. This is no less true in putting together this book. I would like to thank Stavros Garantziotis, MD, at the National Institute of Environmental Health Sciences, who I called upon to serve as co-editor and unhesitatingly accepted the role. I also would like to express our gratitude to the contributing authors who dedicated time out of very busy schedules to lend their expertise. The book could not fulfill its purpose without their generosity. Lastly, I would like to thank Eugenia Judson and Margaret Moore at Springer publishing, who patiently supported this project from inception to finish (in the face of an untimely viral pandemic). We hope that the information summarized in this book serve as a foundation for next-step studies and perhaps attract new investigators into pursuing the next frontier of this exciting research area.

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Part I
Principles and Tools of Respiratory Microbiome Investigation

Chapter 1
Approaches to Sampling the Respiratory Microbiome

Robert P. Dickson

Introduction

Despite sustained and growing interest in lung microbiome research in the past decade, and despite accumulating evidence that lung immunity and pathophysiology are calibrated by local interactions with lung microbiota [1, 2], the broader human microbiome field has remained dominated by gut microbiome studies. A major driver of this difference is the comparative ease of sampling lower gastrointestinal specimens (via stool or rectal swabs) as compared to the intrinsic difficulties of sampling the lower respiratory tract. While sampling of the gut microbiome is fraught with its own (often overlooked) methodological challenges (e.g. the variable bacterial density of rectal swabs, discordance between the communities detected in stool and those detected in colonic and cecal specimens [3–5], the confounding influence of unmeasured factors such as colonic transit time [6]), the issue of accurately sampling the respiratory microbiome has been a central concern since the dawn of the field [7–9].

The unique challenges of sampling the respiratory microbiome stem from the unique anatomy and ecology of the respiratory tract as compared to the relative simplicity of the gut. Whereas the gut is essentially a single long lumen, the lungs represent a fractally dividing tree, with airways expanding to the 70 m^2 surface area

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of alveoli [10]. Whereas bacterial transit thorugh the gut is relatively simple and unidirectional (cranial to caudal), bacterial movement in the lungs is bidirectional and tidal: a constant trickle of microbial immigration via microaspiration and inhalation [11], a steady efflux via mucociliary clearance and cough [12–14]. The anatomic site of interest in the respiratory tract is highly contingent on the disease process being studied: whereas alveolar sampling may be crucial for interstitial and alveolar processes such as fibrotic lung disease [15–17] and acute lung injury [18, 19], airway sampling (via protected brushings) may be more biologically appropriate for airway diseases such as asthma [20, 21]. There is simply no "gold standard" specimen for studying the respiratory microbiome, as the sampling approach much be tailored to the question at hand, balancing practical considerations (e.g. need for sedation, invasiveness of approach) with those related to anatomy (which site is most pertinent to the disease process in question?) and procedural concerns (what is the microbial biomass of the specimen and how vulnerable is it to sequencing contamination?).

This chapter will provide an overview of commonly used sampling approaches for studying the respiratory microbiome. Advantages and limitations of each will be discussed, and practical advice for distinguishing signal from noise will be provided.

Our Evolving Understanding of the Bacterial Topography of the Respiratory Tract

Thoughtful selection of sampling strategy must be informed by an understanding of the unique microbial ecology of the respiratory tract and the recent revolution in our understanding of it. For roughly a century [13], the dogma that "the normal lung is free from bacteria" was promulgated (usually without citation) in textbooks [22]. Yet in the earliest published studies of lung microbiology, published in the decades following the dawn of germ theory (1898–1929), investigators actually reported that viable bacteria could be cultured from the lungs of humans and large animals [23, 24], that lung bacteria in animals resembled the microbial content of nearby bedding and hay [25], that thousands of viable bacteria are inhaled each hour [26], and that subclinical microaspiration of upper airway secretions is common among healthy adults [27]. Before the dogma of lung sterility took hold, the working theory was instead that lungs are under constant exposure to the microorganisms of inhaled air and the upper respiratory tract [25].

Three common conceptual errors gave rise to the misperception of lung sterility. Firstly, investigators misapplied and misinterpreted clinical microbiology testing. Culture-based protocols used in clinical microbiology laboratories were never meant to detect the normal respiratory bacteria present in health, but rather the high quantities of pathogenic bacteria present during acute infections. These protocols disadvantage growth of anaerobes and bacteria with optimal growth temperatures below 37°C, which encompasses much of the bacteria now routinely detected in

lower respiratory tract specimens. Identification of pharyngeal-associated taxa is routinely dismissed as “oral contamination” or not reported. Yet when a variety of culture conditions are applied to lung specimens from healthy subjects, more than 60% of taxonomic groups identified by sequencing can be identified by culture [28].

A second conceptual error that contributed to confusion regarding lung sterility was the inappropriate conflation of the ideas of “ecological contiguity” and “contamination.” Considerable taxonomic overlap exists between the bacteria detected in sterilely obtained lower respiratory tract specimens and those of the oropharynx [28, 29]. This should be unsurprising, as the lungs are in direct mucosal communication with the upper respiratory tract, which serves as their primary source of microbial immigration in health. Likewise, the mucociliary escalator is constantly transporting lower respiratory tract bacteria (and their DNA) cranially to the pharynx so that they may be swallowed or expectorated. Yet despite these anatomic considerations, the presence of pharynx-associated taxa in lower respiratory tract specimens was inappropriately dismissed as “contamination.” As a parallel comparison, gastric microbiota more strongly resemble pharyngeal microbiota than do lung microbiota from the same subjects [14], yet their identity is never dismissed as mere contamination.

The third conceptual error was the confusion of “absence of resident microbiota” with sterility. We have known since the 1920s that the lungs are under constant bombardment by bacteria from inhaled air [30] and subclinical microaspiration of oropharyngeal contents [11, 27, 31, 32]. We have also known that these bacteria are subject to constant turnover thanks to our innate and adaptive host defenses and the mucociliary escalator. Yet the absence of resident, reproducing bacteria (such as we expect in the lower gut) is not at all equivalent to sterility: microbial and nonmicrobial communities can be defined exclusively by the balance of immigration and elimination [12, 33, 34], even if we were to assume the absence of resident, reproducing “colonizers.” Claiming that healthy lungs are “free of bacteria” because they lack resident, reproducing communities would be equivalent to saying that college campuses or nursing homes are “free of humans” because no on-site reproduction of their population occurs. Experimental human and animal studies in the past decade have confirmed that however transient lung microbiota may be in health, they exert a biologically significant impact on the lung immune response [1, 2, 35].

We now know that healthy lung bacteria, measured by quantification of bacterial DNA, are roughly 100-fold lower in concentration than oral bacteria [14], which in turn are orders of magnitude less dense than lower gut bacteria. Yet this small bacterial burden is roughly 100-fold greater than the DNA present in reagent control specimens [14]. Lung microbiota appear to be more dynamic and less temporally stable than lower gut microbiota: while no human study to date has compared serial lung microbiome specimens in healthy volunteers, animal studies (equine and murine) reveal that lung communities are rapidly influenced by changes in environment [2, 36], more so than concurrently sampled lower gut communities [2]. A recent human study revealed the first evidence to date that the “microbiome of the built environment” exerts an influence on the community composition of lower respiratory tract microbiota [37, 38]. Bacterial density (and similarity of

communities to pharyngeal microbiota) is greater at the main carina than in more proximal or distal airway mucosa [12], likely reflecting the gravitational influence of subclinical microaspiration in healthy (upright) humans. While spatial variation has been reported in lung bacterial communities of patients with advanced lung disease [8], spatial composition of bacterial communities within the lungs of healthy volunteers is relatively homogeneous [39]. Intra-subject variation (communities within the lungs of healthy volunteers) is far less than inter-subject variation (communities collected at the same anatomic site across different volunteers) [39]. In their community composition, lung bacteria of healthy volunteers resemble oropharyngeal communities, whether sampled via sputum, bronchoalveolar lavage, or protected specimen brushing [7, 12, 28, 40]. This similarity between lung and pharyngeal microbiota is stronger than in humans than in mice and horses [2, 36], reflecting our unique respiratory anatomy (with a reservoir of microbiota - the oropharynx - positioned gravitationally above our larynx and distal airways).

Sources of "Noise" in Sampling the Respiratory Microbiome

Put broadly, there are two main sources of false signal in respiratory microbiome studies: *sampling contamination* (e.g. contamination of lower respiratory tract specimens by upper respiratory tract microbiota introduced via the sampling process) and *sequencing contamination* (e.g. bacterial DNA present in laboratory reagents). Given the stark gradient in bacterial density present in the respiratory tract (greatest in the pharynx and lowest in the alveoli), sampling strategies are often faced with a trade-off between the two: high-biomass specimens such as sputum are less vulnerable to sequencing contamination but more vulnerable to sampling contamination attributable to pharyngeal microbiota, whereas low biomass specimens such as bronchoalveolar lavage are less vulnerable to sampling contamination but more vulnerable to sequencing (reagent) contamination. The two concerns must be balanced with the scientific question motivating the study (i.e. what is the anatomic site of interest), as well as practicalities such as cost, convenience, and patient safety.

Sampling Contamination Passage of lower respiratory tract specimens through the upper respiratory tract introduces the risk of contamination via pharyngeal bacteria. Yet rigorously controlled bronchoscopic studies have demonstrated that pharyngeal bacteria exert minimal influence on bronchoalveolar lavage and protected specimen brushing specimens [12, 39], so long as appropriate precautions are used (e.g. minimizing suction through the main channel prior to lavage). Sputum, of course, inevitably contains an admixture of upper and lower respiratory tract microbiota, though the relative distribution of the two is highly contingent on volume, technique, and patients' ability to clear their own airway secretions. While surgical excision of lung tissue bypasses the upper respiratory tract and in principle should avoid pharyngeal contamination, the sedation and endotracheal intubation of surgical procedures introduces a risk of peri-intubation aspiration of pharyngeal con-

tents. Thus while sampling contamination can be minimized, its exclusion cannot be guaranteed even under optimal sampling conditions.

While both sampling contamination and sequencing contamination are major methodological challenges, neither has prevented the field from generating findings that are microbiologically coherent, clinically and biologically significant, and reproducible across laboratories and approaches. While specific details of sampling strategies are discussed below, common features of study design apply regardless of specimen type [41]: minimize systematic bias both in sampling and processing of specimens; include copious negative controls (both procedural and sequencing); transparently report all sequencing results, including those of negative controls; confirm the reality of key findings via contextual plausibility, reproduced experiments, and validation using complementary assays.

Commonly Used Approaches to Sampling the Respiratory Microbiome

Bronchoalveolar Lavage

While more invasive, expensive, and time-consuming than collection of sputum, bronchoalveolar lavage offers numerous advantages as a strategy for sampling the lower respiratory tract. While theoretically vulnerable to sampling contamination due to passage via the upper respiratory tract, multiple studies have provided reassuring evidence that the actual risk of pharyngeal contamination via bronchoscopy is minor. "Bronchoscope contamination controls" (protected specimen brushes passed through the bronchoscope channel into the empty airway lumen without touching the airway walls) are indistinguishable in their bacterial burden and community composition from negative control specimens [12], indicating that the microbial signal detected in subsequent lavage specimens is not attributable to pharyngeal contamination. When serial lavages are performed during the same bronchoscopy, there is no decay in bacterial signal (as would be expected if each subsequent lavage was diluting the fixed signal introduced via pharyngeal contamination) [12, 35, 39]. Despite the divergent microbiota of the mouth and nose, the route of bronchoscope insertion (oral vs. nasal) has no detectable influence on BAL microbiota [29, 33, 42]. Finally, bronchoscopically detected microbial communities have unambiguous *correlative significance*: their burden and community structure correlate with variation in alveolar immunity [1, 35] and gene expression [43] and are predictive of disease outcome [18, 44, 45].

Bronchoalveolar lavage typically requires conscious sedation as well as topical anesthesia of the upper respiratory tract. The scope is advanced via the mouth or nose through the larynx, then "wedged" in an airway at a segmental or subsegmental orifice. Sterile saline is instilled and then suctioned. Bronchoalveolar lavage samples not merely the distal alveoli but also the mucosal surface of all small

airways distal to the wedged bronchoscope. The volume of returned lavage fluid is highly variable, which introduces a potential source of procedural variation to microbiome analysis. This variation is offset by two aspects of commonly used microbiome analyses: (1) relative abundance data in microbiome studies is compositional rather than absolute (and thus not influenced by absolute differences) and (2) variation in bacterial DNA burden across specimens is far greater than relative variation in lavage volumes (e.g. 100- to 1000-fold variation in bacterial burden vs. twofold to fivefold variation in lavage return) [14, 46]. Suctioning should be avoided until the time of lavage in order to minimize contamination of the sampling channel. The most commonly sampled anatomic site is the right middle lobe, though (as discussed above) intra-subject variation in bacterial communities is significantly less than that of inter-subject variation [39]. A single lavage per volunteer/patient has been considered adequate for bronchoscopic lung microbiome studies to date. While the volume of instilled saline is variable across practitioners, our practice is to use serial 40 mL aliquots of saline (generally up to 120 mL) until roughly 20 mL of lavage fluid is returned. "Whole" bronchoalveolar lavage fluid (which has not been clarified with a low-speed, short-duration centrifugation cell-removal step) is the most commonly used bronchoalveolar lavage specimen, though use of acellular fluid is acceptable and has been successfully used [1, 18]. The cell removal step does alter detected community composition and decreases the diversity of detected bacteria, and cell-free bronchoalveolar lavage fluid is likely at heightened risk of sequencing contamination [47, 48].

Procedural control specimens should be collected at the time of bronchoscopy and included as controls for sequencing and quantification. For human and large animal bronchoscopic studies, we routinely collect oral rinse, nasal rinse or swab, sterile (unlavaged) saline, and scope rinse (saline instilled through the pre-lavage bronchoscope). The bacterial burden of scope rinse specimens is extremely small, and the risk of scope contamination across patients/volunteers is theoretical and has not been demonstrated. This theoretical risk can be obviated via the use of single-use, disposable bronchoscopes, though it has not been established that this is necessary.

Protected Specimen Brushing

Another bronchoscopic approach that has been used effectively in microbiome studies is protected specimen brushing. Similar bronchoscopic technique is used as in bronchoalveolar lavage, but rather than wedging the scope, a sterile brush enclosed in a protective sheath is advanced through the bronchoscope and only exposed moments before sampling [49]. Whereas bronchoalveolar lavage samples distal alveoli (evidenced by the return of alveolar macrophages), protected specimen brushing provides direct mucosal sampling of the airways. This has proven useful in

the study of airway diseases such as asthma [7, 20, 50]. The surface area sampled by a single protected specimen brush is far less than that sampled by bronchoalveolar lavage, resulting in predictably lower bacterial load [12], and added caution should be taken to address sequencing contamination (discussed above).

Sputum

Sputum is probably the most commonly used specimen in respiratory microbiome studies, and is especially common in chronic airway diseases such as cystic fibrosis, bronchiectasis, and COPD. Advantages to sputum over bronchoscopic specimens include its low cost, the ability to compare serially collected specimens from the same subject, and the lack of need for sedation or anesthesia. Additionally, culture-based analysis of sputum has been a hallmark of respiratory microbiology for decades, providing abundant clinical context for culture-independent findings.

A central and unresolved controversy in the field has been how representative sputum specimens are of lower respiratory tract microbiota given the predictable admixture of salivary and pharyngeal microbiota with lower respiratory tract secretions. While communities detected in concurrently sampled sputum and lower respiratory tract microbiota differ in their community composition [51], sputum communities do consistently differ from concurrently sampled oral wash specimens [52]. While the relative representation of lower respiratory tract microbiota in sputum is variable across specimens and impossible to quantify, bacterial communities detected in sputum have been successfully correlated with important clinical features such as severity of airway obstruction [53, 54], intensity of airway inflammation [53, 55], response to viral infection [56], frequency of subsequent exacerbations [57, 58], and mortality [59]. Whereas clinical microbiology protocols typically have a quality threshold for ensuring the lower respiratory tract origin of sputum specimens (e.g. absence of squamous epithelial cells), no consensus has emerged for sequencing-based characterization. Not all patients with airway disease routinely produce sputum, and induction using nebulized saline has been used successfully [56, 60]. The sputum induction process does introduce potential sources of sampling and sequencing variation, and it is unwise to directly compare induced and expectorated sputum specimens in the same study without considering this potential source of confounding and bias [61–63].

An important difference between sputum and bronchoalveolar lavage specimens is sputum's viscosity, which can impede efforts to pipette and extract DNA. Commercially available additives can be used to facilitate processing, including dithiothreitol and lysostaphin. These techniques can introduce taxonomic bias to sequencing results [64, 65]. While acceptable (and commonly used), these approaches should be used consistently across specimens within a given analysis to avoid false grouping due to a batch effect of procedural variation.

Tracheal Aspirate

A sampling strategy uniquely applicable to mechanically ventilated patients (in intensive care units or operating rooms) is the use of tracheal aspirates. This technique is performed using suctioning via patients' endotracheal tube, and represents a safe, repeatable, and inexpensive means of accessing the lower respiratory tract. Unlike bronchoalveolar lavage (which is used clinically for specific diagnostic and therapeutic purposes and typically requires additional consent on the part of patients and their surrogates), collection of tracheal aspirates is generally considered a routine element of ICU care and introduces no additional risk or cost beyond standard practice. While tracheal aspirates do not access the alveolar space (as does bronchoalveolar lavage), they do provide an accessible opportunity to study an admixture of lower and upper respiratory tract microbiota that has proven useful for the study of the dynamics of respiratory microbiota in critically ill patients, with established correlative and prognostic significance [66–68]. As an example of cross-specimen coherence, a near-identical taxonomic group (an *Enterobacteriaceae*-classified operational taxonomic unit) has been associated with the acute respiratory distress syndrome in studies using both endotracheal aspirates [67] and miniature bronchoalveolar lavage specimens [18]. A key advantage to this approach is the ability to sample repeatedly in the same subject, permitting longitudinal assessment of respiratory microbiota. Whereas tracheal aspirates perform similarly to bronchoalveolar lavage specimens in the clinical (culture-based) diagnosis of ventilator-associated pneumonia [69], no study to date using microbiome methods has directly compared communities in paired bronchoalveolar lavage and tracheal aspirates from the same patients.

Upper Respiratory Tract Swabs

Several studies have used swabs of the posterior nasopharynx or throat to characterize respiratory microbiota [70–72]. These specimens have mainly been restricted to the study of microbiota in children, who typically cannot produce sputum on demand and are infrequently studied using bronchoscopy. There is considerable clinical precedent for the use of throat and pharyngeal swabs for detection of pathogens in cystic fibrosis and suspected viral infections. While these specimens do not sample lower respiratory tract microbiota, their use can be supported given the ecologic contiguity of the lower and upper respiratory tract, the continual passage of lower respiratory tract microbiota to the upper respiratory tract via mucociliary clearance, and the predilection of many lower respiratory tract pathogens to colonize the upper respiratory tract prior to infection. Like sputum and tracheal aspirates, swabs are inexpensive and repeatable, facilitating longitudinal study of the same subjects. Swabs should not be assumed to be high-biomass, and appropriate precautions should be taken to minimize and account for sampling and sequencing contamination [73].

Surgically Resected and Explanted Lung Tissue

At first blush, surgically excised lung tissue may seem like a "gold standard" approach to sampling the lower respiratory tract given the unique opportunity to bypass the upper respiratory tract (theoretically side-stepping concerns of sampling contamination). Yet this sampling approach has multiple limitations, both practical and methodological: (1) patients undergoing lung resection uniformly receive prophylactic antibiotics prior to surgery [74, 75], which alters lung microbiota even in healthy subjects [2]; (2) lung resections require general anesthesia and endotracheal intubation, introducing the risk of periprocedural aspiration of pharyngeal microbiota; (3) lung resections are rarely, if ever, performed on patients with normal lungs, and often have more than one concurrent pulmonary process (e.g. lung cancer *and* chronic obstructive pulmonary disease); (4) even moderately sized tissue snips are predominantly comprised of interstitium and contain small alveolar and airway surface area (which is well-sampled by bronchoalveolar lavage); (5) many fixatives used in the preservation of lung tissue (such as formaldehyde) degrade DNA [76]; (6) the cost and risk of surgical excision preclude sampling in any setting other than clinically indicated procedures. For these reasons, and despite its theoretical advantages, resected lung tissue has only uncommonly been used in lung microbiome studies [77–79]. A more commonly used approach has been via sampling of explanted lung tissue, most often from patients undergoing lung transplantation for end-stage lung disease [8, 44, 77, 79–82]. While many of the above caveats still apply, the explantation of whole lungs permits controlled, serial sampling across the entire biogeography of the airways and alveoli.

Uncommonly Used Noninvasive Sampling Approaches

Though host- and pathogen-derived volatile organic compounds can be detected in exhaled breath [83, 84], and while host-derived biomolecules can be quantified in exhaled breath condensate [85], to date no study has convincingly showed that exhaled breath (or its condensate) contains sufficient bacterial DNA for microbiome analysis. A dedicated study found that bacterial DNA in exhaled breath condensate is indistinguishable from that of reagent control specimens, and varies across specimen replicates (reflecting the under-appreciated influence of sequencing stochasticity as a source of false signal) [86]. Similarly, a sheep study demonstrated that exhaled breath condensate contains far less bacterial DNA than matched protected specimen brushings, with taxonomically divergent taxa [87]. While ventilator expiratory circuit filters (including heat and moisture exchange filters) contain bacterial DNA reflective of culture-identified pathogens in ventilator-associated pneumonia [88], their utility in non-pneumonia conditions (in which lung bacterial biomass is considerably lower) has yet to be established.

Sampling the Respiratory Microbiome in Animal Experiments

Though still lagging behind human respiratory microbiome studies and gut microbiome animal studies, numerous studies have begun to use animal models to interrogate the host–microbiome interface within the respiratory tract. Animal studies using modeling of lung microbiota have been published using mice [2, 19, 89–100], monkeys [101], sheep [102, 103], pigs [104, 105], and horses [36]. While potentially powerful in answering questions that would be impossible or impractical to study in humans (e.g. controlled exposures and disease models, definitive sampling of matched tissues from the same animal, control over host genomics), animal models of respiratory microbiota have intrinsic limitations, largely due to anatomic, physiologic, and microbiologic differences across species. Yet animal modeling in gut microbiome studies has proven fruitful, and the ability to control animal microbiota using germ-free and gnotobiotic facilities can address some cross-species limitations. In fact, several prominent and provocative recent studies have suggested that the immune systems of conventional laboratory mice are "humanized" via restoration of their microbiota to that of wild (non-laboratory) mice [106–108]. Thus failure to address variation in microbiota (both gut and lung) is likely already latently confounding non-microbiome animal studies.

For large animal studies, the same approaches discussed above for human studies should be considered. Porcine and simian models have been used as to model of human disease [101], and sheep have been used to study lung microbiome biogeography and to test whether exhaled breath condensate can be used as a specimen for lung microbiota [87, 102, 103]. Adult horses have been studied to determine the influence of environmental microbiota on the community composition of the lung microbiome [36].

In contrast, unique considerations apply to study of murine lung microbiota. Murine lungs are extremely small (~0.2 g) compared to human lungs (~1300 g), and thus contain even less microbial biomass than already-low human specimens. While several studies of bronchoalveolar lavage of murine lungs have been published [89, 94, 96], most murine lung microbiome studies have used homogenized lung tissue. The taxa identified in lavage fluid and homogenized lung tissue differ from each other [89], attributable likely both to differences in total bacterial biomass as well as which compartment is being sampled. Some groups have pooled multiple specimens when reporting murine lavage fluid data [96], which increases bacterial biomass but precludes analysis of specimen-to-specimen variation. Our practice is to use homogenized lung tissue (using at least one complete lung) for each mouse, an approach that reliably yields bacterial signal distinct from that of negative controls and correlated with variation in host innate immunity [2]. In a direct comparison of whole lung tissue and bronchoalveolar lavage fluid from mice, whole lung tissue contained more bacterial DNA and had distinct community composition relative to negative control specimens when compared to lavage fluid, while bacterial DNA detected in lavage fluid was minimally different from those of procedural, reagent, and sequencing controls [109]. Murine studies are highly vulnerable to the

confounding influence of cohousing, attributable in part to coprophagy as well as the shared influence of microbiota in bedding and chow. The lung microbiota of healthy adult mice cluster by their environment (by cage, by shipment, and by vendor), and lung microbiota from different vendors converge when cohoused [2]. Thus whenever possible, mice should not be housed according to experimental exposure, as invariably the "batch effect" of cohousing will confound analysis of the effect of the exposure on lung microbiota.

Summary

Numerous sampling approaches for studying the respiratory microbiome have proven useful, yet no single specimen type is without limitations. Investigators should tailor their sampling strategy to the scientific question at hand, which includes consideration of anatomic site of interest, relative risk of sampling and sequencing contamination, and practicalities such as cost and safety. Thoughtful collection and analysis of sampling and sequencing controls will facilitate downstream discrimination of signal and noise in sequencing-generated microbiome data.

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Chapter 2
Concepts, Tools, and Methodologic Considerations for Lung Microbiome Research

John R. Erb-Downward

Culture-Independent Microbiology and the New Microbial Tree of Life

In 1977 Carl Woese and George Fox changed our understanding of how cells evolved (1). Studying the low sedimentation rate fragment of the ribosome (the 16S rRNA from the prokaryotes and the 18S rRNA from the eukaryotes), they cataloged sequences from multiple prokaryotic and eukaryotic organisms. When they compared them, they found that the evolutionary tree had not two main Domains, but three, which we now know to be Bacteria, Archea, and Eukaryota. What is more, this new branch was more closely related to eukaryotic organisms than bacteria. We now know that the bacterial 16S rRNA molecule was perfect for this study, as it is a ubiquitous molecule, about 1500 basepairs in length, composed of regions that are relatively constant in all bacterial phyla and nine variable regions where the sequences vary greatly between individual bacterial species. Soon after, with the invention of PCR and the molecular revolution that followed, it was realized that this molecule could be used to identify lineages of bacteria without the need of phenotypic characterization; thus, culture-independent microbiology was born. To date, 16S rRNA gene characterization has been the principal workhorse in the study of the bacterial component of various microbiomes. The lung holds a special place amongst these microbiomes because it was believed to be sterile for so long and without culture-independent techniques, most people would still believe that was the case. In the sections that follow we will discuss the tools, techniques, and methodologic considerations that have been employed in the characterization of microbes in the lung.

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Clinical Microbiology Labs

Of all the enterprises studying microbes in the lung, the clinical microbiology lab has been doing it the longest. When we talk about studying the lung microbiome, the clinical microbiology lab is often skipped over because of the difference in focus, but it is worth discussing what they do and how they do it because their results shaped over a century of believing that the lungs were a sterile site within the body. Clinical microbiology labs focus on the identification of pathogens from clinical samples. As such, the techniques that are generally employed focus on identification of single organisms. Primary among the techniques employed has been bacterial culture, though there has been a shift in more recent years to molecular techniques.

Culture For the vast majority of the twentieth century the tools of bacterial analysis were culture and a microscope. Bacterial culture methods are by far the most common and properly performed, precise methods used for bacterial identification. The ability to selectively enrich the presence of specific members of a microbial community through careful control of metabolic resources or environmental growth conditions makes culture the gold standard for determining whether a bacterium is living in an environment. That said, the converse is not true: if a bacterium does not grow when cultured, then it did not exist in the environment. The fallacy of this line of thinking is that the exacting growth requirements for many species of bacteria cause culture to have an extremely high false negative rate, unless the conditions for growth are known. This has had a tremendous impact in the study of the lung microbiota because frequent results from healthy individuals were no growth in culture or the growth of bacteria associated with the oral cavity (believed to be procedural contamination). Thus, the myth of the sterile lung was created and perpetuated.

MALDI-TOF Beginning with the culturing microorganisms from a clinical sample, many labs utilize mass spectroscopy to identify microbes. Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry works by overlaying a sample containing $>10^5$ Colony Forming Units (CFU) of the microbe of interest with matrix which is then subjected to soft ionization using a UV-laser. This enables analysis of large biomolecules and produces a pattern of protein masses from the sample. These patterns are then matched to a pattern in a database of known microbes (2). Provided sufficient cells can be cultured, this provides an efficient and cost-effective means of microbial identification. PCR-based molecular tests for specific pathogens have also found their way into common practice, but again the purpose of these assays is to identify a pathogenic microbe within the lungs, not characterize what is thought of as the lung microbiome.

Community Characterization with the 16S rRNA Gene

Techniques that involve using the 16S rRNA gene for microbial identification broadly fall into two classes: community characterization and individual identification. The former represent methods that first PCR amplify the 16S rRNA genes in a sample, then fragment them using restriction enzymes to reveal fragment patterns which amount to fingerprints of the microbial community. Individual identification methods, like it sounds, allow one to isolate a specific sequence and use specific portions of the sequence (in combination with large databases) to give a taxonomic identity to the organism from which the sequence originated. This can be done via sequencing or hybridization. Oftentimes the bacteria don't have a good representation within the databases. To get around this problem, sequences are frequently given temporary, or operational, taxonomies. That is to say sequences are put together with other sequences that look similar, and the bin containing those sequences is given a number. Comparisons can then be made between the various bins to learn more about individuals and their community. The details of analysis methods will be discussed in future chapters. In this section we will focus on methods for acquiring information about our microbiomes.

DGGE and TGGE Some of the earlier methods of community characterization were denaturing gradient gel electrophoresis (DGGE) (3) and temperature gradient gel electrophoresis (TGGE) (4). The principle behind these techniques is that small fragments of DNA run on an acrylimide gel across a gradient of either increasing denaturant, or increasing temperature, will produce bands which can differentiate single base pair differences between fragments. A short time after this discovery, it was realized that 16S rRNA gene fragments could also be separated from each other in this way (5) and that this pattern, or fingerprint, could be used to characterize even very complex bacterial communities. Furthermore, individual species could be identified by running isolates along with the community samples. The principal drawbacks of these methods were that they were very operator-dependent, and results between runs could be difficult to quantitatively compare (6, 7).

T-RFLP Terminal restriction fragment length polymorphism (T-RFLP) is a method that employed the automated and more reproducible nature of capillary sequencers to determine the sizes of fluorescently labeled DNA fragments (8). In community analysis the 16S rRNA genes are PCR amplified using primers containing a fluorescent label on the 5′-primer. The products would then be degraded using a restriction enzyme so that only one fluorescently labeled fragment would remain for each bacterial species. Running these fragments on a capillary sequencer would then result in a pattern where the fragment length indicated the bacterial species, and the intensity of a fragment correlated with the abundance of the species. Like DGGE, a fingerprint of the community could be obtained from this method; however, many

more samples could be run, and more reproducibly, than gradient gel electrophoresis methods. Had it not been for the massive throughput offered by next-generation sequencing, this technique likely would have become a mainstay for community analysis.

Identity Methods: Hybridization The idea behind hybridization approaches is that the generation of a primer against a portion of the 16S rRNA gene provides a measure of taxonomic specificity. There are two common hybridization-based methods used in microbiome studies: the Phylochip and fluorescence in situ hybridization (FISH).

Prior to next-generation sequencing the technology for getting the maximum amount of data out of a sample was the DNA Microarray. These assays were performed by hybridizing fluorescently labeled DNA or cDNA products of a sample to an array of thousands of probes specific for certain genes anchored to a chip or slide. The amount of fluorescence measured was then proportional to the amount of hybridization that had taken place. The Phylochip is a specialized version of microarray technology where the probes were 16S rRNA segments taken from a database of known 16S rRNA gene sequences that vary between 0% and 3% (9). Fluorescence intensity indicated abundance of a particular organism (9, 10). By leveraging the previous decade's work refining how to get the best data possible from microarray, Phylochip studies were able to perform comprehensive bacterial surveys when next-generation sequencing technologies were just becoming viable (11–14).

Fluorescence in situ hybridization (FISH) for bacterial species is a technique where fluorescent probes for specific portions of the 16S rRNA gene are used in tandem with fluorescence microscopy to identify organisms and where they are located spatially (15). While the probes utilized can be very finely tuned to identify various taxonomic ranges, the strength of this technique is not as a means of surveying the population, but rather the ability to show the existence of a specific organism, in its environment, in a culture-independent manner.

Identity Methods: Sequencing

The sequencing-based methods leverage two technologies that have been advanced over the past 2–3 decades: the sequencing of DNA and the creation of vast searchable databases into which all of this information has been stored and annotated. At the time of writing (mid 2021), the National Center for Biotechnology Information (NCBI) lists genomes for more than 12,000 Eukaryotic organisms, 265,000 Prokaryotic organisms, and 39,000 viruses. The SILVA ribosomal database lists almost 9.5 million aligned small subunit (SSU) 16S/18S rRNA gene sequences. All of this work has been done within the 25 years since the first genome of a living organism was sequenced (16). Much of the explosion of genomic information is due to what is frequently referred to as next-generation sequencing (NGS). Because

sequencing-based methods are the most commonly used of all the methods mentioned here, it is worth taking a moment to understand them more completely.

To fully appreciate the scale of change brought about, and the limitations that it imposed, it is important to understand a bit about how sequencing actually works. The idea behind Sanger sequencing was that a base analog, such as a dideoxynucleotide, caused a polymerase enzyme to stop replicating at that point. Therefore, a mix containing the proper proportions of regular and dideoxy analogs would produce an incomplete copy at every position where the analog could be incorporated along the sequence. Four such reactions (one for each nucleotide) would be created and the products would then be run out on an acrylamide gel capable of separating DNA fragments that differed in length by a single base pair. A researcher would read across the lanes to determine which base had stopped the sequence at each position to determine the sequence. What are commonly referred to as Sanger sequencing machines were an automated version of this process introduced by Applied Biosystems in 1986 where the base analogs were fluorescently labeled (a different color for each base), and the fragments run past a fluorescence detector using capillary gel electrophoresis (17). The fluorescent peaks indicated the base at the position. A superposition of the peaks from all color channels revealed the DNA sequence itself. As a side note, the automation of this method marks the shift when DNA based technologies became inextricably tied to computers. Very little changed with how sequencing was performed until 2005 when the first NGS sequencing machine hit the market.

At this point the increases in computational power, coupled with advances in nanofabrication (which facilitated the creation of plates where enzymatic reactions could be carried out in two million picoliter volume wells), and dense CCD arrays for capturing high-resolution images, gave birth to the 454 pyrosequencer. The underlying principle of this technology was that as DNA is synthesized a pyrophosphate is released after the addition of each base. This pyrophosphate could then be turned into a substrate for a light producing luciferase at a 1:1 ratio. The emitted light could be captured by the camera, and computers would perform image analysis across the two million wells to determine the base (when light had been emitted) and the intensity (in the case of a run of a single base) for a particular well. Suddenly, running a sequencing machine could produce hundreds of thousands of sequences per run instead of one. The effect of this increase in efficiency due to an increase in throughput cannot be overstated as it would be the deciding factor in determining which NGS technology would dominate the field.

The second NGS system to hit the market was the Solexa, a clever system that did away with the concept of individual wells, instead simply binding DNA fragments to capture oligonucleotides spread out across a glass surface. Each captured fragment would then undergo a few rounds of local amplification resulting in a small cluster of identical sequences. Next, a process referred to as sequencing by synthesis was performed. Here, nucleotides (each with a unique fluorescent label), would be added and a polymerase would copy a single base and pause. The label not only identifies the nucleotide incorporated but also keeps the polymerase from proceeding. A laser would next be injected into the glass substrate at an angle that

causes the generation of an evanescent field. This field is used to excite the fluorophores, but because the intensity of an evanescent field decays exponentially as a function of the distance from the substrate, only the fluorophores which are close to the surface as part of the growing chain fluoresce. A high-resolution camera would then take a color picture of all emitted light, the enzyme would remove the fluorophore, and the whole process would repeat itself. The practical upshot of the differences in these systems was that while the length of the Solexa product was tiny (30 bp), the throughput was 10-100× that of the 454 sequencer. Both companies were bought out (454 by Roche and Solexa by Illumina) and over the next few years the technologies improved immensely (increases in read lengths and throughput), but the throughput of the 454 system was always bound by the number of wells that could be squeezed onto a plate. Because of this, Roche discontinued the 454, and Illumina systems became the dominant force in NGS and the new gold standard in sequencing. This is not to say that advances in sequencing technologies have stopped.

Recent years have seen the development of so-called third and fourth generation sequencers. Perhaps the most promising of these, partially because it still seems like a piece of science fiction, are the Oxford Nanopore sequencers. These sequencers sequence native DNA, cDNA, or RNA by passing the nucleotide sequence through a modified bacterial pore placed in an artificial membrane and measuring the characteristic current changes as each nucleotide passes the pore. These sequencers are capable of sequencing megabase size DNA fragments in real-time in a device the size of a stapler. Of course, all of this data would be useless without computational tools and databases to make sense out of it. However, because of the tremendous work done many an unsung programmer and bioinformatician, we now have the capacity to take virtually any nucleotide sequence produced by modern day methods, and be able to assign some kind of information as to function, the identity of the organism of interest or at the very least the closest evolutionary neighbors. As a whole, sequencing provides a very broad umbrella under which there are many methods of characterizing a microbial community.

Colony PCR This is a quick technique whereby a sample is taken, usually with a toothpick, from a bacterial colony growing on an agar plate and the whole 16S rRNA gene is amplified using universal primers (often without need of a separate DNA extraction step). The PCR product is usually sent off to be sequenced using Sanger sequencing. When the sequence is returned, the closest match is determined using 16S rRNA gene databases like SILVA. Because this method amplifies the whole 16S rRNA gene, the identity obtained is the best at speciation using this marker gene (to the extent that the bacterial species can be differentiated by 16S rRNA sequence).

16S rRNA Gene Surveys As has already been mentioned, the 16S rRNA gene is a central tool for culture-independent microbiology because with it we can easily track bacterial phylogeny with the intent of identifying bacteria. To do this in the context of a bacterial community, the pool of 16S rRNA genes are PCR amplified

and the products sequenced to sample the community. The depth to which a sample is sequenced is treated as the number of individuals examined within the community. The ability to accurately assign a taxonomy to a sequence depends on (1) how well represented are members of that taxonomy within the database, and (2) how much variation exists between bacterial taxa across the gene region being sequenced (recall that different technologies sequence different length products, and of these only long-read platforms like the nanopore sequencer are capable of sequencing the entire 16S rRNA gene). To address the limitations of databases, sequences are often assigned to operational taxonomic units (OTUs) using a method that groups sequences by sequence similarity. To address the limitations of sequencing length, portions of the 16S rRNA gene have been chosen whose rate of evolution mirrors the rate of the whole gene itself (18).

The biggest problem for trying to perform 16S rRNA gene surveys of bacterial communities before NGS was that Sanger sequencing systems could not separate out the sequences. The solution was to clone each 16S rRNA gene amplicon into a plasmid vector and transform *E. coli* with the plasmids. When done correctly a single *E. coli* would pick up a single plasmid. Then individual transformants would be selected, grown up to sufficient density, the plasmids purified, and sent for sequencing. It is easy to imagine the logistical challenge of trying to adequately sample the community from a single sample let alone a cohort worth of samples. While this method has been largely made irrelevant by NGS, it is worth noting that the foundational work to demonstrating bacterial communities in the lungs of healthy individuals using culture-free methods did not use NGS, but did so using clone libraries (19).

At the time of writing, Illumina NGS systems are the de facto standard for high-throughput sequencing. In addition to being able to sequence multiple sequences at a time, researchers routinely multiplex their samples by adding a short index (or barcode) to the forward and reverse primers. This enables them to sort which samples the sequences came from after the sequencing is complete. In this way, up to 384 samples are commonly sequenced while maintaining an average sampling depth of around 20,000 reads (20). The field has not stopped there though, as newer technologies such as the nanopore are capable of both high-throughput and full-length 16S rRNA gene sequencing. Additionally, the small form factor of the MinION should be ideal for in-the-field measurements of bacterial communities. How this will change the field remains to be seen, as it is increasingly being recognized that the sequencing of a single gene frequently falls short of what we need to know about the identity of the bacteria that we detect.

Metagenomics: Metagenomics refers to the sequencing of all of the genomic information in a sample, regardless of origin. It is a completely untargeted approach that can best be described as “sequence everything, and let God sort it out.” God in this case is, of course, the resident bioinformatician who weaves observed reality together from millions of random DNA fragments from hundreds of samples. There are several kinds of information that can be taken from metagenomic data: (1) who is present (classification), (2) what do they have the capacity to do (pathway assembly), and (3) new genomes (genome assembly). The first is simultaneously

straightforward and difficult. As has been mentioned, the vast databases of genomic information have made it relatively simple to have genomic information against which to compare; however, short reads are difficult to differentiate from taxonomically close neighbors because there is not enough information per read. A common strategy for dealing with this involves looking at the frequency of usage of sets of nucleotides (k-mers) that are typical of particular taxa (21–23). It should be noted that both k-mer based and alignment-based methods produce more accurate results if either short reads are joined together into contiguous sequences, or if the reads themselves are long (e.g. nanopore). Pathway assembly attempts to map the reads onto known metabolic and biosynthetic pathways. Once mapped, comparisons can be made in the potential of different communities to utilize resources, to produce a specific metabolite (e.g., butyrate) or even to assess the resistance to antibiotics based on the antimicrobial resistance genes identified (24–26). Constructing genomes from metagenomic data could be considered the "Holy Grail" of culture-independent microbiology because it all but completely eliminates the need for culture. However, while to get a result is relatively straightforward, to make sure that result is correct takes an extraordinary amount of work (27, 28).

There are but two inherent problems that prevent metagenomics becoming the standard: cost and depth. NGS is relatively inexpensive because it can be multiplexed, but the extent to which it can be multiplexed is dependent upon the sequencing depth that one needs. With metagenomics, one is no longer only sequencing just what one is interested in, one is sequencing everything. If, in a clinical sample, one suspects that there are 1000 human cells to every bacterial cell, the number that needs to be considered for sequencing is closer to 10^6 to 1 (that is to say there is a million times more human genetic information than bacterial). This is because one human cell has around 6 gigabases of DNA compared to the average bacterial genome of 3.87 megabases (29). This means that detecting smaller genomic targets (e.g. viruses and phages) requires either an extraordinary depth, overwhelming infection, or sample preparation methods designed around enrichment of viruses. That said, recent work into using nanopore long read sequencers has produced some tantalizing glimpses into how metagenomics might be used in the future for diagnosis of infections (30, 31).

Each of the tools that have been described here have been used to characterize microbes in the lungs. This microbiome has been shown to play a role in many inflammatory and obstructive lung diseases such as asthma, interstitial pulmonary fibrosis (IPF), and chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (32, 33). Yet studying the lung microbiome is difficult – despite the common set of tools. This is because the lung is hard to sample, and because it is a low-bacterial biomass environment. The former of these is rather obvious in that there is one way into the lungs and one way out. Bronchoscopy-obtained samples yield the most reproducible results, but require the aforementioned bronchoscopy. While this does provide a large logistical hurdle for studying the lung microbiome, it has been much easier to deal with than the latter. Studying a low-bacterial biomass environment requires special protocols and methods for assessing the data that is generated. It is on these points that the next sections will focus.

Moving Beyond the Myth of the Sterile Lung

The microbiome of the lung holds a special place in microbiome research; both historically and methodologically. Historically, the lungs were considered sterile organs under normal, healthy, conditions. This belief was largely an extension of the inability to grow in culture bacterial organisms from samples that came from the lungs or if bacterial growth was achieved, the organisms looked like oral flora that were therefore discounted as procedural contamination. This belief persisted until 2010 when Markus Hilty and colleagues published an article detailing disorder in the microbial communities of airways of asthmatics and individuals with COPD relative to healthy controls, using culture-independent methods (19). This initial finding was quickly supported by others and sparked an intense debate over whether the lung microbiome existed or was simply an artifact of bronchoscopic contamination. This debate has now been settled, and it is now broadly accepted that there exists lung microbiome that is seeded by microaspiration from the oropharynx (34). This microbiome has been demonstrated to have important immunologic consequences, despite maintaining a fairly low bacterial load (35–38). The lung microbiome is best described as having low bacterial biomass; although, this is frequently shortened to just “low-biomass” despite copious amounts of human “biomass” being present. This differentiates it from most host-associated microbiomes in that the ratio of bacteria to host cells is so much lower. Being a low-biomass sample, there are several methodological considerations that need to be addressed and differ from the standard analysis of, say, a fecal microbiome. The first is how the 16S rRNA gene is amplified from samples.

Standard 16S rRNA gene amplification protocols frequently fail when confronted with the relatively high amount of host DNA at the same time as low concentrations of bacterial DNA. This appears to result in off target binding of primers and amplification of host DNA, and has been a consistent problem whether trying to amplify the full-length 16S rRNA gene or portion of the gene (e.g. V4 hypervariable region), as is common with most next-generation sequencing methods. The solution for this problem has been to tweak the amplification protocol to offer an opportunity for the low abundance bacterial DNA to be amplified (39, 40). One particularly successful strategy transforms the standard protocol into a touchdown PCR protocol that forces high affinity primer binding during the early cycles to avoid off target binding when this is most likely, followed by gradually relaxing the stringency until the normal cycling conditions are reached for the second half of the amplification reaction (40). In this way, low amounts of bacterial DNA can be amplified despite the high host background. The cost of this increased sensitivity for the detection of low abundance bacterial signal is just that: the detection of low abundance bacterial signal – everywhere. In a seminal study by Salter, S. and colleagues it was demonstrated that a low level of bacterial DNA contamination exists in the reagents that are used to purify and isolate bacterial DNA (41). These low-level contaminants do not cause problems where high-bacterial biomass are concerned, as the contaminants are out competed by the high amount of sample specific

DNA; however, for low bacterial biomass samples, the contaminants compete almost on equal footing with the sample specific bacterial signal. This brings us to the second methodological consideration critical for any lung microbiome study: contamination and controls.

For the purposes of this discussion the word "contamination" should be thought of as the presence of a non-sample associated bacterial signal in a sample. The reason for such specificity in what seems like a straightforward concept is that at least four separate sources of non-sample bacterial signal have been identified to date, and all are generally considered to fall under the umbrella of contamination although their etiologies are very different. The first of these sources is, as was described above, the presence of small amounts of bacterial DNA in laboratory reagents. This is a ubiquitous problem and is not specific to any one vendor. This means that a DNA purification of a negative control (i.e. a procedural purification that lacks any sample) can still produce a microbial community. Moreover, the kind of microbial community tends to vary by reagent batch, meaning that a laboratory contamination will look different depending on which lab performed the DNA preparation and sequencing. There are sparingly few remedies for this, although one moderately effective approach has been to use classical DNA purification methods that employ organic solvents (e.g. phenol-chloroform) which are much less likely to contain microbial DNA. Generally, the only way to be sure of one's data is through the careful selection of controls, as will be discussed momentarily. The second type of contamination is where the samples are contaminated from another sample source. While much of this is preventable through good laboratory techniques, it has been suggested that liquid-handling robots may contaminate samples by inadvertently spreading micro-droplets beyond the targeted well (42). The third form of contamination is not a physical contamination, but rather a misreading by the sequencer of the DNA barcode that defines which reads go with which samples. The result is that reads from one sample are assigned to a different sample, which results in the "presence" of non-sample DNA in the sample (43, 44). The fourth kind of contamination is the appearance of a stochastic noise when sequencing insufficient amounts of DNA (45). This noise can look like and be classified as real microbial signal; however, these signals do not replicate between technical replicates and are thus separable from the physical contaminations mentioned above. Taken together, contamination represents a significant problem to interpretation of low-biomass data, but not an insurmountable one, as we will now discuss.

Given the contamination issues discussed above, the key to a well-designed lung microbiome study is in the controls. To deal with reagent contamination one needs to have separate controls for critical reagents and inputs. For example, a bronchoscopy study would need to sample the saline before it is passed through the bronchoscope, saline passed through the bronchoscope before the bronchoscope touches the study subject, the results of a DNA purification where no sample was added, and elution buffer alone would all be the necessary controls to interpret the results of a BAL from a single individual. Barcode swapping is best dealt with at the level of the sequencing facility. The usage of dual-barcode (barcodes on both the forward and reverse primers) or unique molecular identifiers (UMIs) has been shown to

dramatically reduce swapping events. Stochastic noise can be dealt with by sample replication, but this is not necessary for all samples. Only samples that contain fewer than 10^5 copies of the 16S rRNA gene are vulnerable to noise (at 10^3 they are likely to be dominated by noise), so whether or not replication is necessary can be determined in advance of sequencing by assessing the levels of the 16S rRNA gene by qPCR or ddPCR (45).

The study of the lung microbiome requires very careful planning and controls. The tools are the same as other areas of microbiome research, but how to get good data from the tools and how to interpret that data is different. But, once all lung-specific considerations are taken into account, lung microbiome research is one of the most interesting areas of research around.

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Chapter 3
The Rest Is Noise: Finding Signals in Lung Microbiome Data Analysis

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Introduction

Finding signal in the presence of noise is a fundamental problem in science [1]. As most microbiome studies to date have focused on characterizing the gut, noise (contamination) is generally of minor relevance, as this body site harbors the largest reservoir of bacterial diversity and biomass in the body. The lung, however, has a significantly lower microbial load, increasing the likelihood of contamination during experimental procedures. Further, lung sampling is generally performed through bronchoscopies, which introduces unavoidable cross-contamination from the upper respiratory tract. Combined, these issues have resulted in important challenges in the analysis and interpretation of lung microbiome data. In addition, while lung bacterial communities have been described more comprehensively, the viral and fungal component of the lower airways remain for the most part ignored. While the analytical issues are similar to those of bacteria, it is important to understand the characteristics of viral and fungal data in the context of lung studies. Finally, our knowledge of the lung microbiome is primarily based on relative abundance data, which cannot estimate bacterial load – an important clinical parameter – and

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introduces important analytical biases. Here, we present an overview of data analysis in lung microbiome studies, including a discussion on the different types of data that can be generated, methods to analyze them, issues associated with low biomass and how to detect contamination, and the important distinction between relative and absolute estimates of microbiome composition. We propose a conceptual pipeline for data analysis in lung microbiome studies that addresses each of these issues, and discuss important next steps for future studies.

Data Types in Lung Microbiome Studies

Amplicon-Based Approaches

Although the healthy lung was traditionally considered sterile, this notion has been challenged by novel molecular, culture-independent approaches which showed that, even in the absence of infection, the lungs contained diverse and dynamic microbial populations [2–4]. The advent of next-generation sequencing (NGS) has revolutionized the microbiological landscape, and allowed the characterization of entire complex microbial communities and their functions without the requirement of culture [5]. NGS typically encompasses three main approaches to microbial community profiling: amplicon-based, shotgun metagenomics, and metatranscriptomics sequencing.

Amplicon-based approaches are based on performing PCR amplification and sequencing of highly conserved ("universal") microbial genes which contain variable regions that allow discrimination of individual taxa (Fig. 3.1). Amplicon-based sequencing studies have focused almost exclusively on the bacterial fraction of the microbiome, through analysis of the 16S subunit of the ribosomal RNA (16S rRNA) gene. This methodology has allowed the description of the healthy lung microbiome [4, 6, 7] and the study of compositional changes during lung and respiratory airways pathologies, including asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis, and after pulmonary transplantation [8–13]. The collection of commensal fungi in an environment (mycobiome), on the other hand, has been less studied despite its important role in human diseases, including respiratory fungal infections and other chronic lung diseases [14–17]. A limited number of studies have applied amplicon-based sequencing to address the mycobiome in the respiratory tract during health and disease, including COPD [15], asthma [16], cystic fibrosis [18], bronchiectasis [19], and in patients undergoing lung transplant [20].

Several pipelines are available for the analysis of amplicon-based data, including mothur [21], RDPipeline [22], or QIIME [23]. Broadly speaking, microbiome analysis involves three main steps: quality control, taxonomic identification, and estimates of diversity. Quality controls generally involve the filtering of raw sequencing reads based on quality scores and their assignment to specific samples based on indexing barcodes, which can also be error-corrected using Hamming or Golay

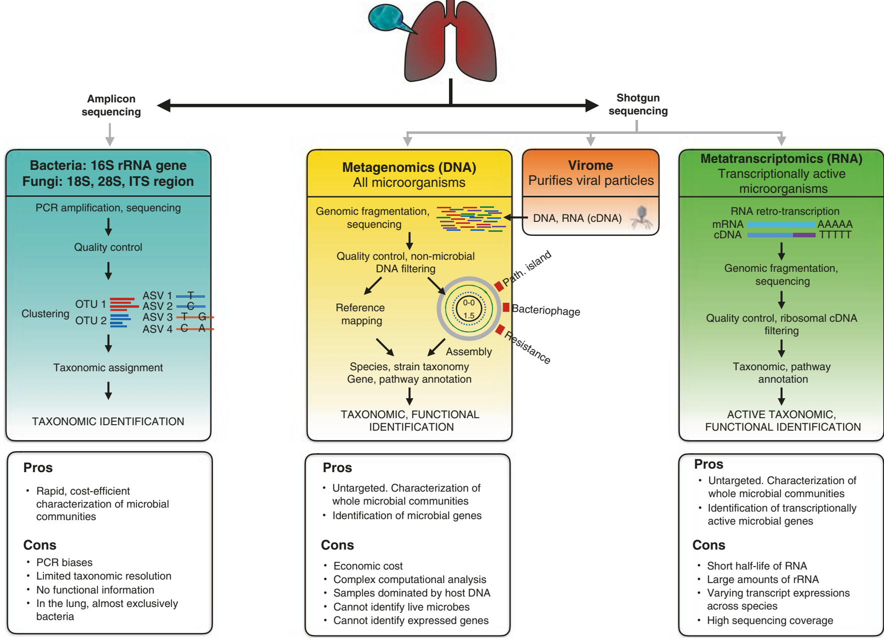

Fig. 3.1 Sequencing-based approaches for lung microbiome data analysis. Main methodologies to characterize the lung microbiome are summarized, including advantages and disadvantages of each approach. (Left panel) Amplicon-based sequencing, used for bacterial and fungal characterization. (Middle panel) Shotgun sequencing: metagenomics. Used primarily for high-resolution taxonomic identification of bacteria (species/strain level), functional characterization, and virome analysis. (Right panel) Shotgun sequencing: metatranscriptomics. Used to identify active components of bacterial communities that are being transcribed and expressed

encoding schemes [24]. Filtered reads are then clustered into Operational Taxonomic Units (OTUs) or “de-noised” prior to their identification as a specific taxon. OTU clustering has traditionally been the standard approach in microbiome studies, where reads are clustered into groups that share the same taxonomic identity (i.e. reads that belong to the same taxon) based on a pre-specified degree of similarity. This process can be performed using reference sequences to which reads are assigned based on their similarity [25], de novo, where reads are clustered using greedy algorithms [26], or with a combination of reference and de novo algorithms [27]. The taxonomy of the OTUs is then either directly inherited from the reference sequence, in case of reference-based clustering, or through similarity search with the representative sequence of each cluster in de novo approaches. Among the most commonly used reference datasets for taxonomic identification are RDP [22], SILVA [28], or Greengenes [29]. Notably, reference-based methods cannot uncover “novel” taxa and are limited by the comprehensiveness of the reference sequence set. These approaches, however, are sufficiently accurate when the environment has been deeply characterized (as it is usually the case with most human body sites) and

they are computationally very efficient. On the other hand, de novo clustering is computationally expensive, as greedy algorithms increase their run time with input size and diversity of the community, but can identify novel taxa not present in reference datasets, which is particularly useful when studying poorly characterized environments [30]. More recently, alternative approaches that do not rely on clustering have been proposed [31–33]. While these methods are theoretically sound and provide finer resolution of the structure of microbial communities, it is unclear whether species-level resolution can be achieved accurately with amplicon-based data alone. Finally, measures of microbial diversity can be calculated using different software packages, including QIIME [23], phyloseq [34], or mothur [21]. Differential enrichment analysis can be performed using methods such as DESeq2 [35] or LEfSe [36] to identify microbial biomarkers.

Shotgun Metagenomics Sequencing and Metatranscriptomics

While amplicon-based approaches have been tremendously fruitful due to their cost-efficiency, they also have important limitations: PCR biases (including differential amplification and chimeric sequences), limited taxonomic resolution below the genus level, and lack of functional information. Shotgun metagenomics sequencing addresses many of these issues by directly sequencing untargeted genomic DNA (Fig. 3.1). While more expensive and data-intensive than amplicon-based techniques, this approach has been successfully applied to study the lung microbiome [17, 37–40]. Because metagenomics does not target a specific gene, this technique can obtain a more complete characterization of microbial communities in the lung, and provide simultaneous estimates of bacteria, fungi, archaea, and viruses by adapting the appropriate experimental protocols for each type of organism. An important consideration with metagenomics sequencing is its ability to produce relevant microbial data in samples that are dominated by host DNA, as it is sometimes the case in lung studies [37]. Because of the large proportion of nonmicrobial data included, a significantly higher sequencing depth is generally required in lung samples to achieve sufficient microbial coverage. The analysis of metagenomics data involves various steps akin to those in amplicon-based studies [41]. Data is initially filtered to remove low quality and nonbacterial reads using mapping tools like fastp [42] or BWA [43]. The remaining reads can then be clustered into contigs, scaffolds, and genomes using tools for metagenomic assembly [44–47], or be profiled for microbial composition, which is computationally less costly than assembling genomes from short-read data [48–51]. More recent tools have also been proposed for the resolution of bacterial strains from metagenomics data [52–54]. Finally, functional characterization of microbial communities can be performed through the identification of specific genes and their annotation to established functions or pathways [55, 56].

An important limitation of shotgun metagenomics is the ability to determine whether genes encoded in a microbial community are being expressed.

Metatranscriptomics overcomes this issue by sequencing microbial RNA in a sample, and provides a more accurate picture of transcriptionally active functions of the microbiome [57, 58]. The importance of investigating the transcriptional profile of the microbiome has been emphasized as recent evidence has shown that expression of microbial genes can change significantly without large alterations in overall community structure [59]. In metatranscriptomic analysis, quality-filtered reads can be assembled into putative transcripts based on known protein sequences [60], or mapped to transcriptionally active microorganisms [48] and annotated for functional and pathway information [55]. As all NGS approaches, metatranscriptomics exhibits its own set of biases and limitations. RNA degrades rapidly, and thus sampling methods are critical to ensure RNA conservation. In addition, ribosomal RNA (rRNA) is highly abundant and must be depleted through molecular methods prior to sequencing or, whenever not possible, during analysis [61]. Sufficient material is required for sequencing, as host RNA can appear in large quantities, and deep sequencing is needed to ensure sufficient coverage of microbial transcripts. Few results exist yet on the transcriptionally active microbiome in the lung [62–64], and future studies will be required to better understand its role in health and disease.

Single-Cell Microbiome Characterization

Methods based on sequencing of mixed populations cannot directly quantify bacterial load nor sort specific subpopulations. Single-cell approaches such as flow cytometry and fluorescence-activated cell sorting (FACS), on the other hand, allow individual microbial cells to be quantified and sorted following specific criteria before additional characterization [65]. This methodology has been utilized to study bacterial communities sorted according to their physiology (size, relative nucleic acid content, membrane integrity) and to the fluorescence emitted by antibodies specifically bound to different human immunoglobulins coating them. When coupled with NGS, it permits the identification of active microbes and the study of host–microbial interactions [66–71]. These approaches are, however, not without limitations. FACS analysis is based on optical properties of the sample and on fluorescent cell labeling, which can lead to differential staining biases. Further, it requires suspension of single cells, but microbes often grow forming colonies or biofilms in nature. Filtering of larger particles and disaggregation through shaking or sonication need to be performed before sorting, and thorough cleaning protocols should be applied to avoid contamination. Importantly, software pipelines for microbiome analysis do not currently provide functionality to work directly with data generated from FACS, which requires the use of ad hoc scripts, which are often not publicly shared and less thoroughly validated than established tools.

Culture methods have been the gold standard used to detect microbial cells in clinical samples, including those from the respiratory tract [72–74]. These methods are less sensitive than sequencing-based approaches, as many strains do not grow (or are fastidious to grow) under laboratory conditions. The use of “culturomics”

(high-throughput isolation and identification of microbes using multiple culture conditions) has gained significant attention in the field [75, 76]. More recently, reverse genomics isolation has been used to capture previously uncultured microorganisms into pure cultures using specific antibodies directed to microbial genomic markers such as predicted surface proteins [77]. Isolated strains are then subsequently identified through whole genome sequencing to discriminate highly related lineages, which also provides genotypic information. This process requires the mapping of reads into existing reference genomes or their assembly into larger contigs, identification of microbial taxonomy, and gene annotation, for which several analysis tools are available [78]. Despite the potential of single-cell methods to study microbial communities, their application to study the lung microbiome is still uncommon, perhaps in part due to the inherent complexity of working with samples of low microbial biomass.

Challenges in Lung Microbiome Characterization and Analysis

The study of the lung microbiome entails numerous challenges. Sampling the human lung is a complex and highly invasive procedure. The two most common approaches to sample the lungs are sputum and bronchoalveolar lavage (BAL), both introducing contaminating material to some extent. Sputum is collected through coughing, which results in samples being enriched in oral microorganisms. BAL, on the other hand, is obtained directly from the lung using a bronchoscope, which may introduce oro-pharyngeal or nasal microbes through its passage to the lungs [6]. While sputum contains material from different niches within the lung, BAL mainly samples the alveoli. Therefore, each sample type captures microbial communities present in different sections of the lungs, and the sampling method should be selected depending on the question addressed.

Besides challenges related to sample acquisition and microbial DNA concentrations, several experimental and analytical issues common to most microbiome research should be considered. Because of variance in microbial cell composition, microorganisms are differently disrupted during nucleic acid extraction procedures. Biases on this initial step can result in under- or overrepresentation of specific taxa, significantly affecting analysis. Correcting for differences in DNA extraction is, however, extremely challenging. Therefore, protocols should be optimized in order to enhance DNA and RNA yields and quality during extraction, for example by combining physical and chemical cell-disrupting methods, including "bead-beating" and the use of specific enzymes (i.e. lysozyme and lysostaphin for bacteria, and lyticase for fungi). Secondly, when using methods that incorporate PCR amplification (such as qPCR or amplicon-based sequencing), the targeted gene is a major factor in determining results. As previously mentioned, the 16S rRNA gene is considered the bacterial marker gene par excellence, and allows the accurate

identification of bacteria from different environments through the amplification and sequencing of its highly conserved regions. It is however important to consider that this gene comprises nine hypervariable regions of varying conservation, and the choice of primer sets used for PCR amplification of those regions may select for (or against) particular groups of microorganisms, significantly affecting the estimations of taxonomic diversity [79, 80]. Furthermore, 16S rRNA gene is present in variable number of copies between bacterial species, which can result in an overestimation of the number of sequences from species with higher gene copy numbers [81]. For example, *Streptococcus pneumoniae*, the leading cause of bacterial pneumonia, has one to five copies of the 16S rRNA gene, while *Haemophilus influenzae*, which can also lead to pneumonia infections, has six to seven copies. On the other hand, species such as *Mycobacterium tuberculosis,* the causative agent of tuberculosis, only have one copy, which could result in underestimation of its relative abundance within a community [82]. Therefore, caution must be taken when trying to compare bacterial quantities between various species in mixed populations. Correcting for 16S rRNA gene copy numbers in microbiome analysis therefore remains an open problem [83]. Some tools have been developed to partially address this issue by using sequence databases and phylogenetic information to obtain more accurate estimates of bacterial populations, including Copyrighter [84], the picante R package and pplacer [85], and tools within the PICRUSt package [86]. However, as these methods rely on reference databases, they still face the problem that less-studied taxa are likely to be missing and would remain biased in their estimates.

Fungal and Viral Data in the Lung Microbiome

Most lung microbiome studies have focused on the bacterial component. However, fungi (mycobiome) and viruses (virome) also play important roles in the ecology of the respiratory tract. The study of fungal communities in the lung is arguably less developed than that of bacteria due to, among others, the lack of standards widely adapted by the community, both experimental and analytical [87]. For example, Charlson and colleagues used commercial DNA extraction kits, bead-beating, targeted the ITS1-ITS2 ribosomal region, and analyzed data clustering at 95.2% similarity in a study of oral and BAL samples from lung transplant patients [20]. Cui and colleagues, on the other hand, used cetyl trimethylammonium bromide for DNA extractions, a 97% identity threshold to cluster 18S ribosomal gene sequences, and a 99% threshold for ITS reads in oral, BAL, and sputum samples obtained from patients with HIV and COPD [15]. These differences in extraction methods and analysis introduce significant biases, which can impact interpretation of results and lead to different conclusions.

Fungal cellular structure and composition, as well as their genetic content, differ significantly from bacteria, which requires protocols to be adapted and optimized for mycobiome studies. The different fungal growth patterns (filamentous vs. yeasts) and the extremely resistant fungal cell wall, which contains chitin, glucans, and

mannans, requires a combination of physical disruption and specific enzymatic lysis to enhance cell disruption and facilitate the extraction of nucleic acids [88, 89]. To date, most mycobiome studies are based on amplicon sequencing [14, 90]. Unlike in bacteria, where the 16S rRNA gene is the gold standard, fungal community composition can be characterized using the 18S rRNA gene, the 28S rRNA gene, and the internal transcribed spacer (ITS) regions located between the 18S and the 28S subunits of the fungal ribosomal gene locus. 18S and 28S rRNA genes are highly conserved across all eukaryotes [91, 92], which makes them a convenient target to detect fungi, but also results in the amplification of other microbial eukaryotic DNA (helminths and protozoa) and, potentially, of human DNA as well. Further, due to their sequence conservation across organisms, these genes offer poor taxonomic resolution, preventing confident annotation at the species level. ITS1 and ITS2 genes, on the other hand, are not part of the conserved ribosomal transcribed regions, being more diverse across eukaryotes, which allows the exclusion of other eukaryotes' DNA through the design of fungal-specific primers [93, 94]. The diversity of the ITS regions also allows a greater depth of taxonomic assignment, often down to species level, making it the preferred region for fungal analysis [94]. However, the lower conservation rate of the ITS sequences results in variations in length and content between fungal species, and depending on the region targeted, results can be skewed towards different fungal phyla [89, 95]. In addition, while 97% sequence similarity is the standard for bacterial 16S rRNA analysis [96], there is no consensus on which threshold should be used in mycobiome analysis, which makes comparison of results across different studies challenging. More recent approaches have proposed the use of amplicon sequence variants (ASVs; also referred to as exact sequence variants, ESVs, or zero-radius OTUs, ZOTUs), individual sequences obtained after removal of spurious reads generated during PCR and sequencing, which distinguish variants differing by as little as one nucleotide [31, 32]. ASVs offer higher granularity than OTUs, and because they do not rely on fixed similarity thresholds, they can partially overcome some of the issues associated with the analysis of ITS data [97].

An additional consideration in mycobiome analysis is the fact that, compared to bacteria, there are significantly less fungal reference genomes available, and repositories often contain redundant sequences, and with incomplete taxonomic assignments [98]. Fungal taxonomy also presents important challenges, and different sexual (teleomorph) and asexual (anamorph) forms of the same fungus are commonly classified as different taxa [99]. As a result, fungal taxonomic classifications based on comparison with databases are prone to errors and can result in very close or identical organisms to be identified as different or fail to assign sequences below family level. Addressing these problems requires the construction of manually curated reference databases for fungal annotation of species [89, 99]. These issues are particularly relevant for shotgun metagenomics sequencing, as reads need to be assembled or mapped into reference genomes. Of note, because lung microbial communities are generally dominated by bacteria, shotgun approaches require very deep coverage to recover fungal sequences [17, 39], sometimes failing to detect

eukaryotic genera [37, 38]. The limitation in available fungal reference genomes further complicates the analysis of mycobiome metagenomics data, as software tools that map reads to the genomes for identification might have low sensitivity.

Given the challenges that studying fungal communities entail, it is perhaps not surprising that the literature of lung mycobiome is sparse. Most studies have used amplicon sequencing [15–19], and only a few could detect fungal reads using metagenomics [17, 39] or metatranscriptomic [62–64] in the lung. Fungi typically found in the oral cavity (such as the ubiquitous *Candida, Cryptococcus, Aspergillus*, *or Fusarium)* are in close contact with the upper airways, and thus, they are likely to be found in the lung [100]. On the other hand, *Pneumocystis* is a common commensal of the airways in healthy conditions, but can cause opportunistic infections (pneumonia) in immunocompromised patients. Similarly, other commensal fungi could lead to opportunistic infections, probably triggered by imbalances in the respiratory microbial communities. The role of fungi in the lung, as well as the mechanisms by which they contribute to (or protect from) disease, therefore remain largely unexplored.

The human respiratory tract is also exposed to and harbors a wide variety of viruses. Although lung infections by specific viruses are well characterized [101], knowledge on the viral communities (virome) is still scarce. The gold standard method for clinical testing of common respiratory viruses is PCR multiplex panels [[102], [103]]. These assays are sensitive and yield rapid results, being a relatively inexpensive diagnostic method. However, PCR-based assays cannot detect viruses not included in the panel or those with "recent" mutations in the targeted region, and cannot be used to subtype viruses [104, 105]. Metagenomics sequencing provides a more sensitive approach to characterize viral communities, including viruses beyond common respiratory pathogens, such as bacteriophages and other human viruses residing in the human lungs. An increasing number of studies have utilized this nontargeted approach to investigate viral infections in the lung [105–107], COPD [108], the effect of smoking [109], cystic fibrosis [110, 111], or in lung transplantation [112, 113]. Shotgun sequencing has also proved useful to survey outbreaks of emergent respiratory viruses [114]. However, as viral DNA (or RNA) only represents a small fraction of the total microbial nucleic acids in sputum and BAL samples, a purification step to isolate the viral particles (VPs) becomes necessary in order to eliminate nonviral elements and permit a deeper characterization of viral communities [106, 110, 115]. Because of the low amounts of DNA extracted from purified VPs, viral sequencing requires deep sequencing efforts, which significantly increase the cost of virome studies. In addition, a large proportion of viral sequences generated from metagenomic sequencing do not align to known reference viruses. This "viral dark matter" reflects the limited representation of viruses in reference sequence databases [116]. Finally, it should be noted that many of the most common respiratory viruses causing infections are RNA viruses, such as the paramyxoviruses, picornaviruses, orthomyxoviruses, or coronaviruses. However, only a few studies have analyzed both DNA and RNA viruses in the lung [106, 113, 115], and many focus exclusively only on viral DNA [109, 110, 112]. This is partially due to

the instability of RNA (which needs to be retrotranscribed to make PCR analysis and/or sequencing possible), which makes working with DNA viruses comparatively easier. Future studies will be required to expand our knowledge of RNA viral communities in the lung.

Analysis of Low Biomass Samples

Next-generation sequencing analyses allow the identification of whole microbial communities and function in a rapid and sensitive manner. This increased sensitivity, however, also increases the likelihood of detecting contaminant DNA not originating from the samples under study. In samples with high biomass, such as feces, contaminants are relatively overridden by the large amount of microbial DNA present in the samples. However, in low biomass microbial environments such as the lung, microbial DNA only accounts for a small proportion of the total DNA, and can be often masked by host DNA and other microbial contaminants [117–119]. This is more likely to occur in amplicon-based studies, that usually employ two cycles of PCR amplifications during library preparation, and thus are more prone to overamplify contaminant DNA [120]. Contamination can compromise the analysis of microbiome data by detecting taxa not truly present in the specimens, which inflates alpha diversity measures, biases relative abundance estimates (see "Compositional Data Analysis"), and impacts differential enrichment analysis, among others. This situation is aggravated when studying the mycobiome or the virome, as fungal species and viral particles are usually outnumbered by their bacterial counterparts.

DNA contamination can arise from different sources. The environment can be an important source of microbial DNA, including body sites close to the sampling site, lab personnel during sample processing, and transmission through air. Laboratory consumables, reagents, and nucleic acid extraction kits also contain trace amounts of DNA, affecting both amplicon-based survey studies and shotgun metagenomics [121]. Additionally, cross-contamination from nearby samples can occur during processing steps, PCR, and libraries preparation. Within the sequencing instrument, cross-contamination between samples analyzed in the same sequencing run is possible, including index switching (where indexes from one sample can randomly "jump" and ligate to other samples) or run-to-run contamination, among others [122].

To minimize the impact of contamination, there are certain measures that researchers should implement during microbiome research [117]. Thorough aseptic conditions should always be kept at the time of sampling and during sample processing in laboratory environments. In addition, use of extraction kits certified as DNA-free is highly recommended. Importantly, strict negative controls should always be analyzed in parallel to the samples to rule out potential contaminations at the time of DNA/RNA extraction, PCR amplification and sequencing. Negative, blank controls, and no-template amplification controls should also be included on each sequencing run. It should be noted that taxa found in negative controls cannot

be simple removed from the analysis, as they may overlap microbes truly present in the samples [123]. Positive controls, on the other hand, are essential to assess biases and detection limits, and can be implemented through the use of "mock" communities, defined mixtures of microorganisms or their DNA, to simulate the composition of a real sample [124, 125]. Mock communities, however, cannot reflect the complexity of an actual microbial community, and might introduce biases due to differences in GC content, Gram staining, or other specific properties of the cells in the mixture. The addition of a specific concentration of external microbial DNA to a sample (spike-in controls) is also useful for sample tracking and absolute quantification of microbial composition, as well as identification of contaminants [126–128]. However, there is a risk of cross-contamination between sequences originating from the positive controls and those found in the samples. Positive controls should therefore be carefully designed with organisms which are unlikely to be present in the samples of interest, although this introduces bias in the correction process [129].

Using the largest possible sample volume is also a good measure to maximize input biomass and minimize biases during analysis. Amplification methods such as Multiple Displacement Amplification (MDA) can increase microbial genomic concentrations by performing whole genome amplification without sequence-specific primers and at constant temperature, which removes the need for a thermocycler [130]. However, MDA can result in amplification biases that may impact subsequent microbial estimates from metagenomic data [131]. Additionally, the use of nonredundant multiple indexing during library preparation can prevent index switching between samples analyzed within the same run [122, 132]. Sequencing reads from pooled libraries can be identified and sorted computationally (demultiplexing) before final data analysis. Finally, an aid to distinguish contaminants from microbiome members while analyzing data is to consider whether the observations represent ecological sense, for example, through examination and comparison with appropriate literature [124].

Similar approaches should be generally applied for lung mycobiome and virome studies. Fungal DNA extraction can also be affected by contamination in reagents and extraction kits even to a higher degree than in bacterial analysis [133, 134], due to lower microbial loads and methodological differences (e.g., more PCR cycles needed in amplicon-based studies). The presence of environmental, contaminant or transient fungi in the lungs can also be problematic. Besides combining sequencing and culture methodologies to study the mycobiome, Fiers and colleagues propose to investigate the potential for host immune responses to fungi, as well as their ability to establish interactions with bacteria, which could be critical to distinguish potential contaminants or transient fungi from commensal mycobiota [135]. This could also be extended to mycobiome analysis in the lungs, where continuous contact with the external environment occurs, and the susceptibility to microbial contamination with fungi originated from the outside environment or other parts of the body (such as the nasal and oral cavities) should be analyzed with especial care.

A problem derived from low biomass in lung samples is the fact that it can lead to lower sequencing coverage. Differences in sequencing depth ("library size") across samples arise mainly due to differential efficiencies during the sequencing

process, rather than reflect real biological divergence. These differences then lead to biased estimates of diversity and composition, which is usually corrected for using rarefaction, which normalizes coverage across samples [136]. However, in studies of low biomass environments this often results in numerous samples not reaching the minimum rarefaction threshold and being dropped from subsequent analysis. Deeper sequencing efforts that yield sufficient sequence coverage of the microbial populations can ameliorate this problem, although at a higher economic cost. Divergence in sequencing depth generally results in an underestimation of microbial composition, as rare taxa can be missed in low-sequence-number samples. This would ultimately lead to differences in beta diversity and lead to biased biological interpretation [137].

Although NGS is currently the gold standard for microbiome studies, there are still important limitations, which are exacerbated when working with low biomass samples, including their susceptibility to contaminations and the need for deep sequencing efforts in order to recover microbial sequences. The use of parallel approaches in combination with DNA-sequencing analysis, such as classic culture-dependent methods or microscopy, and other molecular methodologies, such as real-time quantitative PCR or flow cytometry, are essential in order to validate the observations from any of the different sequencing platforms and help to rule out potential contaminants. The need for developing standardized laboratory and bioinformatic methods that allow researchers to differentiate true positive signals in low biomass samples is also apparent. New methods are being developed to more accurately process low-biomass samples, by establishing protocols from sampling to statistical analysis [138]. Future advancements in the field are guaranteed, which will enhance the efficiency and accuracy of lung microbiome studies, and reduce costs and informatic efforts.

Identifying and Removing Contaminants

As noted in previous sections, analysis of lung microbiome data is significantly hindered by the presence of contaminants. This can take the form of either host or microbial contamination, and is often a consequence of the low microbial biomass found in the lung. Unlike other human niches generally rich in microorganisms (such as the gut or the oral cavity), the lung is naturally colonized by a much lower amount of them, which results in a high host/microbial DNA ratio. This makes the use of methods to deplete human DNA or enrich microbial genomes desirable for lung microbiome studies, especially when addressing fungi and viruses. Computational identification of human DNA sequences can be performed by mapping the reads against the human reference genome, using tools such as Bowtie [139] or BMTagger (ftp://ftp.ncbi.nlm.nih.gov/pub/agarwala/bmtagger/).

Detecting microbial contamination is, however, more challenging. It is important to distinguish experimental contamination, which originates in reagents, during sample acquisition and processing, or as a result of sequencing, from biological contamination that might arise from nearby tissues. Lung studies are particularly

prone to the latter form, as microaspiration can result in microbes from the upper airways drifting into the lung. While it is evident that these microbes are not true commensals of the lower airways, the question remains as to whether it is always the same type of microbes that move from the upper to the lower airways (i.e. the microaspiration process is driven by certain patterns and is not totally stochastic), and whether their presence in the lung is physiologically relevant or not. Because this is, in our opinion, an unresolved issue in lung microbiome research, we strongly advocate for the collection of samples from the upper airways that can be used to remove such contamination if the study hypothesis so requires. Experimental contamination, on the other hand, is always a source of bias that should be removed from the data. Below we explore the analytical options for dealing with contaminants, regardless of their origin, and discuss caveats of these approaches and areas in which we feel further research is still needed.

A first and naïve approach used in some studies is the subtraction of microbial taxa found in negative controls [140, 141] (or in samples from other body sites that might act as contamination sources, such as the upper airways) from true samples. While this method is the most sensitive and can remove all potential contaminants captured through negative controls, it is also the least specific, as these taxa could also truly be part of the lung microbiome. In addition, and because lung microbiome samples often have low coverage, complete removal of such taxa can lead to samples not having sufficient sequencing depth to pass rarefaction thresholds (although see "Compositional Data Analysis" for approaches that do not require rarefaction).

More sophisticated approaches can be used to tag the potential origin of samples using source tracking approaches [142]. These approaches consider multiple possible environmental sources for different microbes, and then ask the question of whether those observed in a specific sample are likely to have originated from that environment or from some other, which would indicate potential contamination. A popular implementation of this general idea can be found in the SourceTracker package [143], a Bayesian approach that considers contamination as a mixture in unknown proportion of microbial sources. An important limitation of these approaches, however, is that they can identify samples as being contaminated and the likelihood of that contamination originating from specific sources, but not whether a given taxa in the sample is only present as a contaminant or could truly be present in the environment from which the sample was acquired. An alternative method has been more recently proposed to detect contamination based on two simple observations: there is a negative correlation between sample DNA concentration and frequency of reads from contaminant taxa, and those sequences are more likely to appear in control samples. The decontam package implements these ideas and has been shown to be effective in low biomass samples [144].

All methods to detect contamination must perform a balancing act between sensitivity and specificity, and it is therefore important to understand limitations of each approach. A recent comparison by Karstens and colleagues [119] demonstrated that while all approaches can identify contaminants, removal of sequences present in control samples can be overly stringent and filters true sequences, therefore reducing power of the study to detect differences. SourceTracker was accurate in identifying contaminants when experimental sources were defined, which makes it

attractive for its use in studies that rigorously sample potential sources of contamination [4]. In the absence of such information, decontam was also found to be effective at tagging contaminant sequences. Regardless of the approach, we emphasize that lung microbiome studies must include analysis of contaminants for results to be interpretable.

Compositional Data Analysis

As with most microbiome studies, lung microbiome data is often compositional: for each sample, the abundance of microbes is expressed as nonnegative values that sum to unity [145]. Any assay that yields only relative abundances of components in a sample is in fact compositional, which has important implication for data analysis, as traditional approaches such as differential analysis or correlation are highly impacted and can often lead to wrong conclusions [146]. This issue is particularly relevant in lung microbiome studies, since the low microbial biomass in specimens leads to higher amounts of contamination than in other body sites. The presence of contaminant taxa in a sample then results in lower estimates of the relative abundance of microbes truly inhabiting the lung, as they are expressed as a fraction of the total number of DNA reads assigned to the sample. A related issue that can lead to compositionality is that of rarefaction: because sequencing coverage is different across samples, estimates of composition and diversity are calculated from a subsampled set of data so that all samples appear to have the same library size [23]. Apart from questions as to whether rarefaction techniques are appropriate given the resulting loss of power [137, 147], the immediate consequence of normalizing sequencing coverage is the induction of compositionality in the data. Various approaches have been proposed to work with compositional data in microbiome studies. A goodness-of-fit to proportionality statistic based on log-ratios has been proposed to address compositionality issues, which can more accurately estimate when variables are proportional [146]. Differential abundance analysis using relative abundance data can also be performed using an approach based on reference frames [148]. Co-occurrence network analysis is also significantly impacted by compositionality, and methods based on the log-ratio transformation have also been proposed [149, 150]. While these methods can significantly reduce the number of false associations, the high prevalence of zeroes in lung microbiome data remains problematic, as approaches based on log-ratios cannot work with them. The use of pseudo-counts to replace zeroes is not without issues [151], and dropping zeroes from analysis –often used for differential analysis– results again in a loss of power.

The issues related to the use of relative abundance data goes beyond the analytical considerations described above. Microbial load is a critical parameter in microbiology and in the study of infectious diseases [152, 153], and sequencing-based methods alone cannot quantify loads (and thus, cannot estimate absolute abundances) of microbial populations. Real-time polymerase chain reaction (qPCR) is a fast and reliable approach to monitor the amplification of a targeted microbial DNA fragment during a PCR in real-time, and can be used for quantification of individual

microbial species, or overall microbial loads by using universal primers. This method, however, cannot discriminate whether DNA was obtained from a viable cell, a dead cell, or whether it was even free DNA. Methods based on fluorescent dyes and cell sorting, on the other hand, allow the simultaneous estimation of microbial load and viability [66], which can provide more accurate characterization of lung microbial communities while at the same time bypass problems related to compositionality of relative abundance data.

Conclusions

Many of the issues found in analysis of microbiome data are exacerbated in lung studies, due to lower microbial biomass, higher prevalence of contaminants, and the still limited number of community-wide standards for sample acquisition, processing, and analysis. We propose a conceptual pipeline for lung microbiome data analysis that summarizes our recommendations and includes main references to software tools (Fig. 3.2). While this is not a step-by-step description, we believe lung microbiome studies should at least incorporate some form of the proposed measures to achieve reliable and robust conclusions. The use of standard analytical approaches

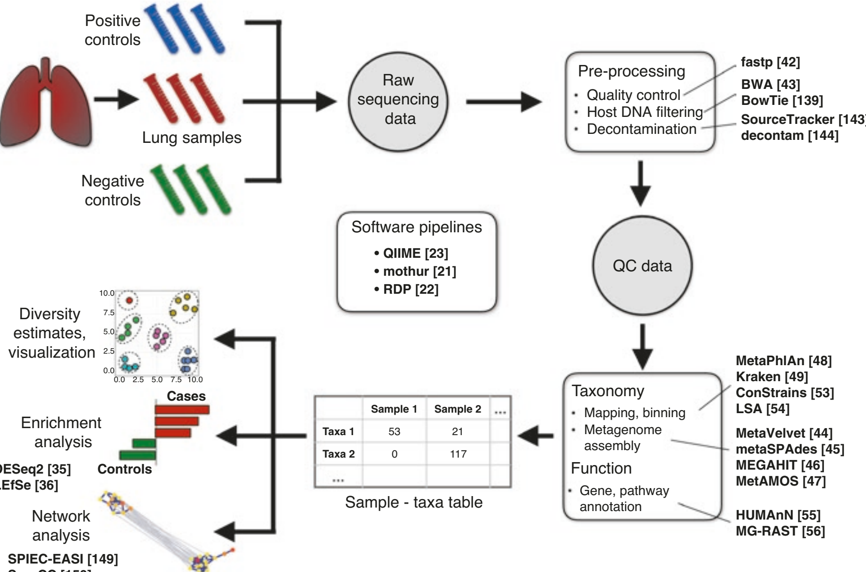

Fig. 3.2 A conceptual pipeline for lung microbiome data analysis. Lung samples are collected together with negative and positive controls, and sequenced using amplicon or shotgun strategies. Raw sequencing data is preprocessed to perform quality control, filtering of host DNA, and decontamination (using positive/negative controls). Data is then mapped or assembled into microbial taxa or genes and pathways to generate contingency tables that can be analyzed for diversity estimates (alpha, beta diversity), visualizations, differential enrichment analysis, or network analysis

will also benefit the research community by facilitating the reuse and comparison of results across different studies.

Numerous open issues remain in lung microbiome characterization and data analysis, in part due to the quick pace at which the underlying technologies used are changing. The use of shotgun metagenomics, for instance, is gaining traction in the field due to its higher resolution as well as the ability to estimate microbial function. There are still important limitations due to the high amount of host DNA that will require optimized methods that remove nonmicrobial cells as a source of contamination. A wide variety of software tools exist for species and strain-level identification, but it is yet unclear what drives the differences among the tools, and the extent to which those inconsistencies might be impacting conclusions across studies. Metatranscriptomics has also gained significant attention recently, although we feel the number of studies in the lung is still limited and there is a notable lack of standards that complicates interpretation. We have not discussed assays such as metabolomics, which can provide additional insights into how lung microbes interact with the host [154]. The problems to address with this data type are in fact common to all microbiome studies: are the observed metabolites endogenous or microbial, can we predict whether metabolites are being produced or consumed by specific microbes, and are there metabolites that require community-level interactions to be synthesized [155–157]. Contamination will continue to be an important consideration in lung microbiome studies, and methods that can predict not only whether samples are contaminated but the extent to which specific taxa are contaminated are highly desirable. It is also important to notice that current studies have generally investigated the bacterial, fungal, and viral components of the lung microbiome as independent entities, which is often not the case, as interactions among them can often have important phenotypic consequences [158, 159]. Finally, we note that lung studies are generally cross-sectional, with only few longitudinal studies. This is an important limitation, as the microbiome changes over time in response to interventions, disease progression, and other factors. Methods that can interrogate temporal lung microbiome data will be important, although they will need to account for irregular sampling frequency and missing data that are characteristic of clinical research.

We have summarized in this chapter current approaches for lung microbiome data analysis, as well as important limitations and issues that, in our opinion, require careful attention when interpreting results. These considerations will help further advance our understanding of how microbial communities in the lung play a role in disease pathogenesis, progression, and outcomes.

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Part II
Applications: Role of the Microbiome in Respiratory Disease

Chapter 4
Allergic Rhinitis and Chronic Rhinosinusitis

Kirsten M. Kloepfer, Arundeep Singh, and Vijay Ramakrishnan

Introduction

The nasal passageway serves as an interface between the external environment and the respiratory tract. During inspiration, the airways are exposed to pollutants, aeroallergens, fungal spores, viruses, and bacteria. The human respiratory tract is divided into two distinct areas: the upper respiratory tract (URT) and the lower respiratory tract (LRT). The URT includes the nasal cavity, turbinates, paranasal sinuses, nasopharynx, and supraglottic larynx, while the LRT is comprised of the subglottic larynx, trachea, bronchi, and bronchioles. This chapter focuses on the sinonasal cavity and its microbiota as it pertains to health, allergic rhinitis, and chronic rhinosinusitis.

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The Upper Airway Microbiome

Early colonization of the URT is determined by the nature of delivery. The microbiota of vaginally delivered infants resemble their mother's vaginal microbiota (*Lactobacillus*, *Prevotella*, *Sneathia*) while those delivered via C-section resemble their mother's skin microbiota (*Staphylococcus*, *Corynebacterium*, *Propionibacterium*) [1]. Associations between specific microbiota profiles and subsequent allergic disease have been established. For example, as infants develop, their URT microbiota becomes dominant in either: *Streptococcus*, *Moraxella*, *Staphylococcus*, *Corynebacterium*, or *Corynebacterium/Dolosigranulum*. Both the *Moraxella* and *Corynebacterium/Dolosigranulum* dominant microbiotas are considered more stable because they are associated with fewer allergic disease outcomes [2]. In addition, these two microbiota profiles are associated with breastfeeding, which often is considered to promote a "healthy" microbiota. In contrast, C-section delivery, male gender, presence of siblings, and daycare attendance have been correlated with dysbiosis (Table 4.1) [3].

The microbiota of the anterior nares in healthy adults is dominated by three phyla: Actinobacteria, Firmicutes, and Proteobacteria [4]. Furthermore, four distinct genera profiles have been shown to dominate the anterior nares (*Staphylococcus, Propionibacterium, Corynebacterium* or *Moraxella*) [5]. The middle meatus microbiota has also been described in healthy adults with the most abundant bacteria observed being *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Propionibacterium acnes* [6]. Evidence suggests that 85% of the nasal community is shared amongst distinct populations and decreases in diversity with age [7].

Table 4.1 Factors that may influence the microbiome of the upper airway

Host factors	Genetics
	Comorbid diagnoses
	Mucosal immunity
Environmental factors	Pollen
	Cigarette smoke
	Diet
	Urban/rural living
	Pollution
	Daycare
	Family size
	Mode of delivery
Medical interventions	Antibiotics
	Nasal steroids
	Nasal irrigation
	Biologics
	Nasal/sinus surgery

Allergic Rhinitis

Pathophysiology of Allergic Rhinitis

Allergic rhinitis (AR) is a type I hypersensitivity reaction mediated primarily by immunoglobulin E (IgE) and mast cells [8]. Inhaled antigens (dust mites, pollen, animal dander) are phagocytized and processed for presentation via MHC II molecules on antigen-presenting cells (dendritic cells, macrophages). The antigen presenting cells migrate to lymph nodes where MHC II interacts with the T-cell receptor on naïve T-helper/CD4$^+$ (T_h) cells with concurrent B7-CD28 interaction co-stimulation. The naïve T_h cells mature to T_h2 cells, releasing numerous cytokines (IL-3, IL-4, IL-5, IL-13, GM-CSF, etc.), which, along with CD40–CD40 ligand interactions, promote B-cell isotype switching to IgE [8–10]. IgE is released into the circulation and binds the FcεRI receptor on mast cells and basophils with high affinity. This process is referred to as allergen sensitization [8–12] (Fig. 4.1).

On ensuing exposures, the antigen binds and cross-links with the antigen-specific IgE resulting in the release of pro-inflammatory molecules and the de novo synthesis of new molecules [9–12]. Preformed histamine is predominantly responsible for the early-stage reaction characterized by runny nasal discharge and sneezing. This occurs through histamine's ability to trigger vasodilation, increase vascular permeability [13], and decrease nasal mucosa epithelial integrity [14]. Simultaneously, mast cells undergo de novo synthesis of arachidonic acid metabolites (prostaglandins, leukotrienes) which contribute to the vasodilation and increased vascular permeability of the early reaction [13].

The cytokines released by T_h2 cells stimulate an influx of inflammatory cells such as basophils, eosinophils, and macrophages to the nasal mucosa, and enhance the maturation of naïve T_h cells to T_h2. Degranulation of the inflammatory cells contribute to the late-stage reaction characterized by nasal congestion. Basophils are functionally similar to mast cells [15], but instead act as the dominant source of histamine in the late reaction [16]. Eosinophils release various inflammatory molecules [15]; however, most important to allergic reactions is the production of leukotriene C_4 thought to be responsible for nasal congestion [17].

IgE levels represent an important distinction between sensitized and nonsensitized individuals and have been positively correlated with allergic symptoms [18]. However, questions remain regarding why some become sensitized while others do not. The hygiene hypothesis postulates that children with fewer infections and otherwise reduced microbial exposures and colonization during childhood have higher occurrences of allergic disorders, especially in westernized nations [19, 20]. The biodiversity hypothesis builds upon this by theorizing that environmental influences modify and disrupt the diversity and quantity of the microbiota contributing to allergic diseases [21]. This represents an important progression as more and more evidence points towards a modulatory role of the microbiota in immune development [22], including the balance of T_h1/T_h2, T_h17 and regulatory T cells of which dysregulation has been associated in the development of allergic disorders [23, 24].

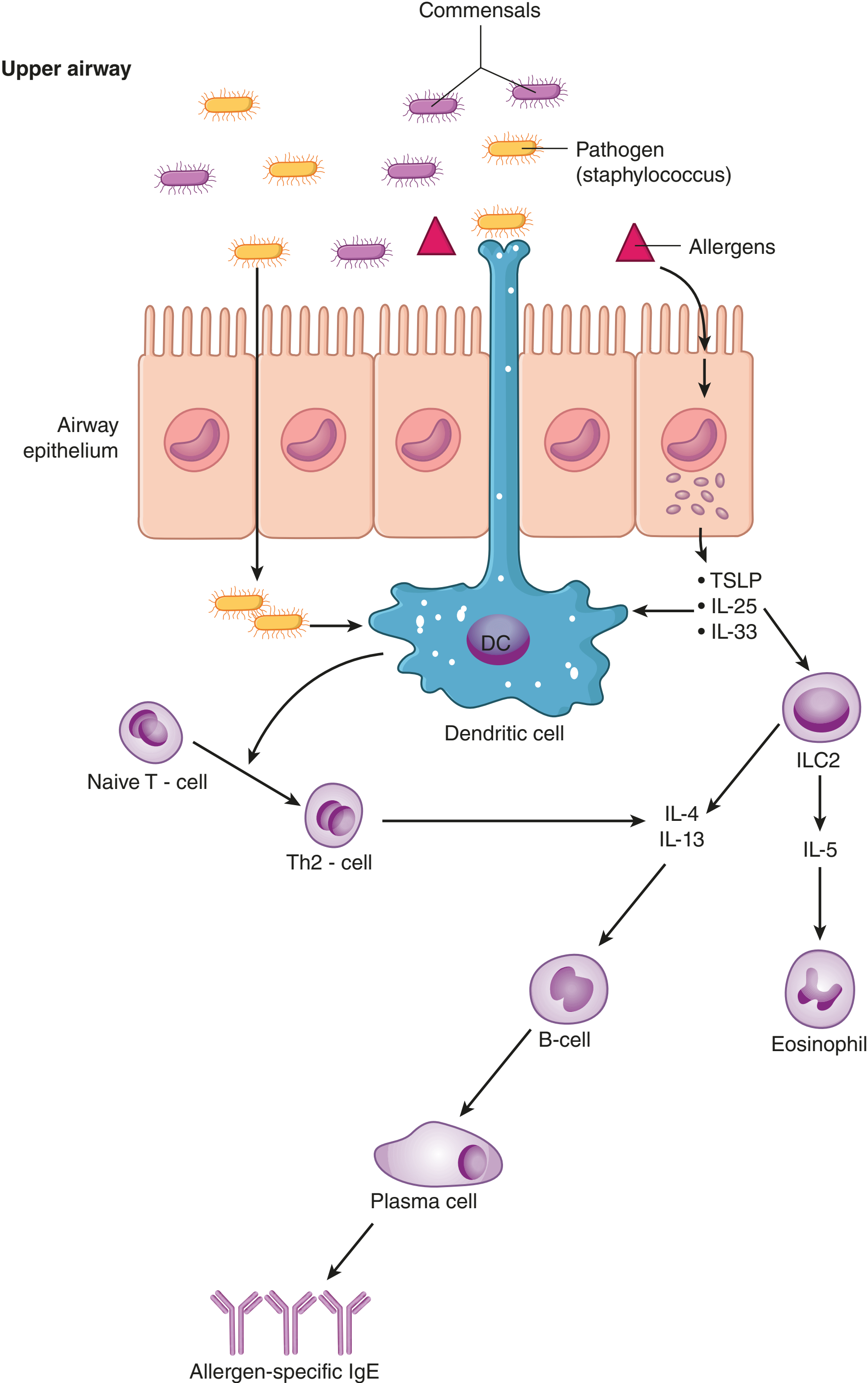

Fig. 4.1 Established inflammatory response to allergens, and potential inflammatory response to bacterial dysbiosis

Given the implication of the microbiota in immune development and allergic disorders, early dysbiosis may be regarded as a potential contributor to disease development.

Dysbiosis in Allergic Rhinitis

Dysbiosis is defined as a change in the microbiota composition including loss of biodiversity, an imbalance in the composition/ratio of microbiota, and as a change in specific lineages composing the microbiota [25]. With its recent implications in human pathologies [26–28] including allergic airway diseases such as AR [28], dysbiosis has been redefined to refer to any change in the composition of commensal microbes that deviates from that found in healthy individuals [29].

Recent evidence links dysbiosis in the upper respiratory tract (URT) to allergic conditions such as AR [30]. Dysbiosis can be seen in the developing infant's nasal microbiome. Compared to healthy infants, infants with rhinitis begin showing decreases in bacterial diversity as early as 3 weeks of age with *Oxalobacteraceae* family (*Proteobacteria* phylum) and *Aerococcaceae* family (*Firmicutes* phylum) predominating [31]. In contrast, *Corynebacteriaceae* family (*Actinobacteria* phylum) and early colonization with *Staphylococcaceae* family (*Firmicutes* phylum) are associated with healthy infants. Further, *Staphylococcus* increases over time until 9 months of age before declining in abundance later in infancy [31]. This decline in healthy infants may illustrate why later high abundances of *Staphylococcus* is linked to AR. Compared to healthy individuals or individuals with low total IgE levels, adults with allergic rhinitis have a low microbial diversity of their inferior turbinate microbiota with a high abundance of *Staphylococcus aureus* (*Firmicutes* phylum) and a low abundance of *Propionibacterium acnes* (*Actinobacteria* phylum) [3]. These findings were associated with high total IgE levels [3], suggesting that allergic rhinitis is associated with dysbiosis. It remains unclear how *Staphylococcus* elicits an allergic response; however, recent evidence points towards the role of superantigens (SEA, SEB, TSST-1) in prompting IgE sensitization [32, 33]. Other *Staphylococcus* proteins (serine-like proteases) have also been implicated in inducing IgE responses [34] or IL-33 activation, an upstream effector of the T_h2 allergic response [35–37].

In humans, several studies have linked antibiotic use, especially early exposure, to allergic disease [38–40]. Early exposure in infants is associated with dysbiosis and/or a less-diverse gut microbiota [41]. However specific mechanisms of action for the selected antibiotic determine which species decrease in abundance with a resulting increase in growth of other non-affected species [42, 43]. The effects depend on the host's microbiome profile [44] and can be long lasting [45]. Antibiotic use has also been shown to cause dysbiosis in the URT [46, 47], though, again, it is likely dependent on the type of antibiotic. Interestingly, use of intranasal steroids in a small pilot study saw increases in microbial diversity secondary to reduction of *Moraxella* and increases in *Actinobacteria* phyla members [48].

Environment exposures can also play a role in microbiota changes. The gut microbiota in those with seasonal allergic rhinitis fluctuates during pollen season

with decreases in *Bifidobacterium*, *Clostridium*, and *Bacteroides* [49, 50] observed. This indicates that the environment, specifically allergen exposure, may play a key role in the changing microbiota. Other environmental exposures such as passive or active cigarette smoke exposure have been associated with the development of allergic disease, particularly in children and adolescents [51]. The consequences of cigarette smoke on nasal microbiota have not been entirely examined, though it has been shown that individuals with COPD have decreased lower airway biodiversity compared to nonsmokers [52].

Local Airway Microbiome vs. Gut Microbiota

In humans, increases in basophil counts contribute to more frequent T_h2-driven allergic inflammation, primarily in the late phase reaction (not excluding the early phase reaction) of AR where basophils are the dominant source of histamine [16]. While a link between increased basophils and the human airway microbiota has not been established, dysbiosis in the gastrointestinal (GI) tract, as a result of antibiotic administration, has been shown to increase IgE concentrations thereby increasing circulating basophil levels in murine models [53]. Antibiotic administration in mice led to alterations in the commensal bacteria population, specifically reductions in *Bacteroidetes* and *Firmicutes* phyla, resulting in increased basophil development in the bone marrow by influencing basophil responsiveness to IL-3 via upregulation of the IL-3 receptor subunit CD123 [53, 54]. This process elucidates a role for the microbiota in mediating or protecting against allergic responses [53].

Dysbiosis of the GI tract has also been seen in AR. Prospective studies examining the gut microbiota of infants from birth through 2 years of age found that dysbiosis of bacteria such as *Escherichia coli*, *Clostridium difficile*, *Bifidobacterium longum*, and *Bacteroides fragilis* precedes the development of allergic diseases [55, 56]. Reduced abundances of the phylum *Firmicutes*, specifically the *Dorea* genus, in commensal populations of the GI tract can also be seen in school-aged children with AR and asthma and are inversely correlated with fecal IgE levels [57]. Much is still not understood about the *Dorea* genus. It belongs to the *Lachnospiraceae* family which includes some probiotics such as Lachnospiraceae and Lachnospira spp. that induce T_{reg} cell and anti-inflammatory molecule production (IL-10 and inducible T-cell co-stimulator) [58, 59]. As opposed to *Dorea*, increased *Ruminoccocus gnavus*, another member of the *Lachnospiraceae* family, has been associated with respiratory allergy occurrence in a twin cohort study [58]. Further investigation into this bacteria found that murine models exposed to *R. gnavus* had an increased release of inflammatory cytokines, particularly interleukin-33 (IL-33), a cytokine implicated in T_h2-mediated allergic responses [35, 36].

Diet has also been shown to influence the microbiota. Three different microbial patterns have been described, with each dominated by either (1) *Bacteroides*, (2) *Prevotella*, or (3) *Ruminococcus* [60]. Dietary intake is linked with dominance of these bacteria. *Bacteroides* is associated with a higher intake of animal protein and

saturated fats (Western diets), whereas *Prevotella* with low meat and greater carbohydrate intake (agrarian, vegetarian/vegan diets) [61]. Western diets have been associated with gut dysbiosis leading to various inflammatory and metabolic conditions [62], and a 2016 study linked low fruit and high meat consumption in preschool aged children to higher risks of developing rhinitis [63]. Despite this, more evidence is needed to link diet to AR. Diet may also play a significant role in gut microbe alterations during urbanization. It has been noted that individuals living in rural environments have greater biodiversity in commensal populations compared to urban dwellers [64] and less occurrences of atopic conditions [65, 66] especially among farm-reared children [67]. As individuals migrate from rural to urban areas, there is westernization of diets that shift microbiomes from *Prevotella*-dominant to *Bacteroides*-dominant [68].

Existing Study Designs for Microbiome Role in Causality or as a Disease Modifier

Causality is difficult to ascertain in human disease. Therefore, murine models, especially germ-free (GF) mice [69], are utilized due to the ability of researchers to directly manipulate and control their environments. Interestingly, GF models have been shown to have higher IgE levels and greater allergic responses [70, 71], another indication of the potential role of the microbiome in allergic response regulation. Recolonization of GF mice with commensal flora of healthy mice (without allergic symptoms) reversed the increased levels of eosinophils and lymphocytes, and reduced the allergic symptoms originally observed in the GF mice [72]. Colonization of GF mice with fecal samples from children with allergic diseases (not AR exclusive) resulted in the GF mice, and their offspring, exhibiting higher T_h17-signatures which shifted their immune development towards atopy [73, 74].

Another way to examine the role of the microbiota in allergic disease is to utilize antibiotics to alter the microbiota. Mice administered with antibiotics have significantly reduced bacterial loads in their GI tract and consequently develop elevations in allergen-specific IgE (and IgG1) levels following sensitization compared to control groups [53, 75]. Given the allergic inflammation observed in the experimental groups, it is likely that altering the microbiota causes allergic inflammation.

If dysbiosis causes AR, then theoretically probiotics that restore the microbiota should decrease allergic reactions. In mice with ovalbumin-sensitization, inoculation with probiotics (*Bifidobacterium breve* and *Lactobacillus plantarum*) reduced eosinophils, ovalbumin-specific IgE and IgG1, and several inflammatory cytokines [70]. Though this study focused on food allergy, a similar reduction in allergic symptoms was noted in mice delivered via C-section who were inoculated with *B. breve* [76].

Further experimental manipulation of microbiota can be induced by diet. Fiber intake has been shown to alter microbiota to contain more fiber-metabolizing

bacterium. Direct alterations of mouse gut microbiota were seen in those fed with high-fiber diets, with better protection against allergic airway inflammation possibly due to greater circulating short-chain fatty acids [77]. This study provides greater insight into how modifications in gut microbiota can influence lung pathologies (lung-gut axis) [78].

To date, a limited number of studies exist, with most studies utilizing murine models. Those focused on humans have small sample sizes, conflicting results, a lack of focus on underlying mechanisms, and rarely focus specifically on AR, making it difficult to prove causality in URT microbiota studies. As focus in this area grows, more definite conclusions about causality between microbiota and AR are likely.

Strength of Associations in Human Studies

While murine models help provide evidence of causality between the microbiota and AR, differences in respiratory and gut anatomy, as well as differing microbial composition limits the translatability of results to human models [79]. Studies examining the upper respiratory tract and GI tract microbiota of study participants after diagnosis of allergic rhinitis reveal alterations in their microbiota. While this suggests that an association exists between microbiota changes and allergic rhinitis, it does not identify the direction of the association (e.g. if the dysbiosis leads to allergic rhinitis or if allergic rhinitis caused the dysbiosis) [3, 57]. Another issue in human studies is self-reporting of symptoms which can differ from individual to individual. To address this, and increase the strength of association, studies have measured IgE levels to evaluate a quantifiable objective measure [53, 57]. IgE is a marker of allergic disease [18], and levels have been linked with early-life colonization of the microbiota [80].

Monitoring early-life colonization in infants prior to the onset of allergic disease allows for better understanding of the timeline of microbiota dysbiosis. For example, a study of two separate populations with vastly dissimilar prevalence of atopic disease examined the intestinal microflora of two-year-old children, noting dysbiotic colonization by *Lactobacilli* and aerobic organisms in those who eventually developed allergic disease [81]. Following subjects and collecting samples immediately after birth until the onset of allergic symptoms strengthens observed associations. Studies collecting fecal samples and monitoring for allergic diseases in infants from birth have found that dysbiosis precedes allergic symptoms and therefore is likely to be partly responsible for AR development [3, 55, 56, 58, 82]. Since the onset of AR is not limited to infancy and childhood [83], a similar longitudinal study following children ages 7–11 until ages 15–20 years noted similar changes in the microbiota preceding allergic symptoms. Specifically, this study reported increased microbial diversity in children who did not develop AR compared to those who did [84].

Modulation of the microbiome provides an additional way of assessing strength of association. If dysbiosis results in the development of AR/allergic diseases, then triggering dysbiosis by use of antibiotics or treating dysbiosis by use of probiotics should increase or decrease the risk of AR in human models, respectively. In the case of antibiotics, use in the first week of life was associated with increased prevalence of AR [85]. In the case of probiotics, human studies have shown efficacy in the treatment of allergic symptoms and restoration of beneficial bacterium (*Bifidobacterium lactis*, *Lactobacillus acidophilus*, *Bacteroides fragilis*) [49, 50]; however, they have not proven effective in prevention of symptoms during pollen season [49].

Considering the associative studies discussed in this section and throughout the chapter, strong, quality evidence exists linking dysbiosis in the microbiota in human models to development of allergic disease. With only a few studies disputing the dysbiosis hypothesis, and support from animal models, it appears that dysbiosis contributes to the development of AR.

Microbiome Modulation Opportunities

Probiotics constitute the most common, and easiest, method of microbiome modulation. Currently, the World Allergy Organization only recommends probiotic use in pregnant women at high risk for having an allergic child; women breastfeeding infants at high risk of developing allergy; and in infants at high risk for developing allergy [86]. Data over the benefits of probiotics, namely *Lactobacillus spp*. and *Bifidobacterium bifidum* [87], in AR have been conflicting despite the observations of decreased quantities of these bacteria in patients with allergic disease [88].

Systematic reviews/meta-analyses of studies utilizing probiotics as a treatment option for AR have shown clinical benefits in nasal symptom reduction and improved quality of life scores, but with the major limitation of study heterogeneity [89, 90]. In prevention studies with probiotics during pregnancy and early infancy, systemic reviews/meta-analyses have shown no significant differences with use of probiotics versus placebo for allergic diseases excluding eczema [91–93]. Additionally, translation of benefits in mice models [94] is often not observed in humans utilizing the same bacterial strains [95], though murine models do provide insight into probiotic mechanisms such as induction of regulatory T cells [94] or altering the T_h1/T_h2 balance [96]. Despite more recent evidence indicating the use of probiotic mixtures [97] and probiotics along with medications [98], the inconsistency of findings suggests that the microbiome is not solely responsible for development of allergic disease or the need to individualize probiotics.

Other modulation opportunities include utilization of prebiotics, dietary supplements, and implementing a healthy lifestyle. Evidence on the use of prebiotics to reduce allergies has also been unclear [99], but in combination with probiotics has been shown to decrease allergy occurrence in infants delivered by C-section [100] and tended to reduce the occurrence of IgE-associated atopic conditions [101]. We

previously discussed the influence of diet on microbiota composition, and the effects of the Western diet [61, 62], fruit and meat consumption [63], and fiber intake [77]. In addition, higher frequencies of vitamin D deficiency have been identified in patients with allergic disease [102], and its supplementation has been shown to reduce allergic symptoms [103, 104]. Further, evidence suggests an association between vitamin D and a microbiome composition predominant in *Prevotella* and low in *Haemophilus* and *Veillonella* [105]. Finally, physical activity has also been shown to influence gut microbiota [106], and increases have been associated with decreased rhinitis symptoms, reduced airway inflammation, decreased IL-4 and eosinophils with increased IL-2 levels [107, 108].

The final consideration for microbiome modulation is fecal microbiota transplant (FMT) . Current findings demonstrate FMT as an effective treatment for recurrent *Clostridium difficile* infections, but variable success for chronic diseases associated with dysbiosis [109]. No human studies have evaluated FMT efficacy in allergic disease; however, transplant studies in mice have shown efficacy in reducing allergic symptoms and associated lab findings [72]. Presently, two clinical trials are evaluating FMT in patients with food allergies and atopic dermatitis, potentially paving the way for FMT studies as treatment for AR.

Chronic Rhinosinusitis

Pathophysiology of Chronic Rhinosinusitis

Chronic rhinosinusitis (CRS) is increasingly recognized for its high personal and societal burden, affecting up to 16% of the population, accounting for over 14 million annual clinic visits in the United States, and resulting in annual disease management costs of $4.3–5.8 billion [110]. Patient reported quality of life (QOL) in CRS is comparable to severe diseases such as congestive heart failure, angina, COPD, and back pain [111, 112].

Historically, the presence of respiratory pathogens (e.g., *S. aureus* and *P. aeruginosa*) and antibiotic resistance found on sinus cultures suggested that CRS was largely an infectious disease, resulting in tremendous antibiotic use, often to no avail. Recently, nuanced understanding of the disease has resulted in a model wherein chronic mucosal inflammation associated with pathological host–microbe interactions may contribute to disease onset, propagation, and/or recalcitrance. In support, multiple 16S rRNA gene surveys of the sinus microbiome have noted loss of commensal organisms, reduced microbial diversity, pathogen presence, and disrupted host–microbe interactions in CRS [110, 113–115]. Colonization of the upper airway mucosae with dense and varied assemblages of microbes, and awareness of their importance to human health and disease has resulted in a paradigm shift from the "one pathogen, one disease" CRS model to the concept of the bacterial community as a functional unit [116, 117]. Microbial ecology and community

interactions appear to govern important roles at the epithelial surface that are critical in CRS, including pathogen exclusion, production of local antimicrobial factors, barrier fortification, immune system development, and metabolic functions [118].

Dysbiosis in Chronic Rhinosinusitis

In *health*, the upper airway is populated by an apparently stable microbiome that is highly individualized [119–122]. Characteristic findings in health include increased bacterial diversity, low abundance of pathogens, and limited anaerobes [6]. *Propionibacteria* and *Corynebacteria* are consistently found in more abundance in health, although precise speciation is subject to technical limitations [6, 123, 124]. Commensal organisms are important to airway homeostasis, as nasal epithelia of germ-free mice display decreased thickness, increased collagen deposition, loss of goblet cells, and loss of nasal-associated lymphoid tissue [125].

In contrast, CRS patients harbor qualitatively different microbial communities [126–129] that may be less stable over time [130]. Given the large interpersonal variability and technical differences across studies, it is not entirely surprising that there is not a single implicated organism in CRS. However, ecological findings such as loss of diversity, preponderance of opportunistic pathogens over commensals, and expansion of anaerobes are consistently observed. Immunological and epithelial barrier defects differ across CRS subtypes [131]; it may be that studies need to examine well-defined CRS subtypes in order to identify findings within overall noise.

In a cohort of 82 subjects, Ramakrishnan and colleagues examined microbiome alterations by phenotype and noted that the presence of polyps was not associated with microbiota alterations in CRS, but CRS patients with asthma or purulence had markedly different microbiota [127]. In this study, the authors did not find differences in alpha diversity indices (richness, evenness, complexity) of CRS patients when compared to controls, but noted that increased diversity was associated with improved surgical outcome, suggesting that a diverse microbiome may be beneficial to restoration of sinus health. Chalermwatanachai and colleagues profiled the microbiota in 41 CRS with nasal polyps (CRSwNP) subjects compared to 18 controls, also finding differences in microbes between the asthmatics and nonasthmatics, and demonstrated that pathogens found in CRS subjects outcompeted *Propionibacterium acnes* in cocultivation experiments [132]. Although one study reported differences in nasal polyps (CRSwNP) compared to control subjects [133], most publications do not observe differences within CRS cohorts driven by polyp status. Specifically examining CRS phenotypes, Hoggard and colleagues did not observe differences unique to CRSwNP, but reported that asthmatics and CRS patients with CF were more likely to exhibit dysbiosis with wide variability in community structure [134]. Similarly, Mahdavinia et al. did not observe nasal polyp status to associate with a unique microbiome in a cross-sectional study of 111 CRS subjects [135].

Cope et al. examined sinus brushings in 59 CRS patients and 10 controls, clustering subjects into four CRS subgroups according to pathogenic microbiota and their predicted functions, as well as host mucosal inflammatory response [136]. The authors observed that one of these four groups had a higher incidence of nasal polyposis, and was defined by a predominance of *Corynebacteria* and increased IL-5. Hoggard et al. reported a cross-sectional analysis on 93 CRS subjects and 17 controls, evaluating microbiota alongside ten tissue cytokines and 6 cell types [137]. The authors identified 8 clusters of patients, strongly segregated by the presence of polyposis, asthma, cytokine profiles, and the loss of healthy bacterial groups. In aggregate, these studies indicate differences in CRS asthmatics, and occasionally in CRSwNP although the effect appears more driven by the presence of asthma. Vandelaar and colleagues retrospectively examined 134 sinus cultures obtained through commercially available microbiome testing, in an attempt to distinguish characteristic microbes found during acute exacerbations (AE-CRS) versus other CRS phenotypes and were not able to uncover differences between groups [138].

Given the thematic observations in these studies, but lack of reproducibility of specific findings, Wagner Mackenzie et al. combined available 16S rRNA sequence data in a 2017 meta-analysis [124]. They concluded that bacterial communities in CRS are dysbiotic and ecological networks of healthy communities were fragmented in the diseased state. Notably, CRS was defined by loss of bacterial diversity, increased dispersion of bacterial communities, and loss of Actinobacteria and *Propionibacteria* that characterize the healthy state.

To understand how these microbial alterations influence host tissue processes, several groups have associated microbiota surveys with host cytokine profiling or tissue function assays. Biswas and colleagues evaluated 23 CRS subjects (8 CRSwNP, 8 CRS without Nasal Polyps (CRSsNP), and 7 cystic fibrosis) and 8 controls, and found two subgroups of CRS patients [139]. One group was characterized by low bacterial diversity and dominance of pathogens such as *Pseudomonas*, *Haemophilus*, and *Achromobacter*. The other group was characterized by preponderance of B cells and CRSwNP, suggesting that integration of microbes with other clinicopathologic features may be required. In a separate report, the authors utilized proteomics and 16S rRNA sequencing of middle meatus swabs in addition to tissue immune cell profiling, to correlate several bacterial taxa in CRS subjects with dyregulation of various host proteins [139].

It is unclear if community microbial function versus a dysfunctional host reaction to microbes is more important. In addition to the bacterial dysbiosis that may be present in CRS, a dysfunctional host reaction to microbiota may also be present. Aurora et al. found minimal differences between the bacterial and fungal microbiomes of CRS versus healthy subjects, but when peripheral leukocytes were exposed to different microbiota, CRS patients produced significantly more IL-5 [140]. Through in situ hybridization, Bachert and colleagues [141] found that *S. aureus* correlated with IL-5 positive CRSwNP, whereas *P. aeruginosa* correlated with nonpolyp CRS (CRSsNP) with high TNF-a. These data suggest that bacteria respond to, and perhaps influence, inflammatory pathways in CRS. Such data indicate that

dysfunctional host immunologic reaction is at least as important as any underlying microbial difference between CRS and healthy states.

Existing Study Designs for Microbiome Role in Causality or as a Disease Modifier

A limitation of existing cross-sectional and case-control study designs is that it remains unclear if the microbiome drives the onset, chronicity, or severity of CRS, the presence of CRS initiates changes in the microbiota, or if both are modified by a lurking or confounding factor [113]. This challenge is frequently observed in CRS study, due to several factors eloquently described in the European Position Paper on Rhinosinusitis and Nasal Polyps [142]: "*Nearly all of the currently conducted human research is performed in patients who already have established disease or controls who do not. While this is useful in identifying unique contributors to the pathophysiology of CRS and subsequent treatments, it does not identify the actual cause of the disease. Currently available animal models are either allergic models or genetically manipulated animals that artificially generate an inflammatory response and again, do not answer the cause of the disease*".

A significant limitation of the field has been the lack of animal models that accurately depict physiological, immunological, and microbiological hallmarks of CRS. The shortcomings of mouse models for CRS limit the generalized approach of recolonization or transfer experiments. As sinonasal epithelial changes are observed in germ-free mice [125], existing mouse models for CRS could be used to test well-defined hypothesis for specific disease subtypes. An early study utilized a mouse model to attempt transfer of human sinus microbes to antibiotic treated mice [123], and observed *C. tuberculostearicum* induction of MUC5AC expression. Further applicability of this model is not clear, as high concentrations of instilled bacteria were required to induce a modest increase in goblet cell hyperplasia, a nonspecific sinonasal response to many stimuli.

In human study, patients recruited for these studies have been subjected to repeated disease interventions prior to study enrollment that are known to alter the microbiome (e.g., antibiotic or steroid therapies). Additionally, the high degree of interpersonal variability is a challenge to overcome given the relatively small cohorts included in studies. Lastly, although there is biologic plausibility for a microbiome role in CRS, there is a generally limited mechanistic understanding of host-microbiome cross-talk in the upper airways governing chronic pro- and anti-inflammatory processes.

Despite these challenges, CRS subtyping and metagenomics approaches do offer support and mechanistic insights. That CRS subtypes exhibit unique microbial signatures suggests that dysbiosis in CRS is not merely a by-product of prior treatments, as these are broadly applied to all CRS patients regardless of phenotype. CRS sub-types, although crudely defined, exhibit distinct immunological,

physiological, and microbiological manifestations [143–145]. Mahdavinia et al. were able to link comorbid allergic rhinitis with the lipopolysaccharide protein biosynthesis pathway using predictive metagenomics, suggesting a functional relevance for the microbiome in atopic CRS, although follow-up experiments were not performed [135].

Strength of Associations in Human Studies

Although CRS presence is frequently associated with altered microbiota and loss of diversity, the causal relationship of the microbiome in CRS is unclear, in part, due to inherent confounders such as CRS subtype-specific disease processes, prior therapies [48, 124, 146, 147], environmental exposures, and host genetics. Notably, existing cross-sectional and case-control human studies have associated microbiota with CRS disease severity or histopathology, beyond mere presence of disease [123, 148].

Unlike allergic rhinitis, large cohort studies prior to and during CRS onset are not feasible given: that CRS is uniquely different in adults (versus children), the inability to identify at-risk individuals, and the need for deeper endoscopically guided sampling. However, intervention study design and associations with outcomes have been performed as another way to support the microbiome's role in CRS. Medical therapies, including use of topical intranasal corticosteroid (INCS) may result in effects that persist well beyond the duration of therapy. Such changes could result from inherent antimicrobial properties or local immune modulation known to occur [48]. Antibiotic administration similarly appears to result in potentially dramatic, although varied, alterations in mucosal bacterial communities [149, 150]. Feazel et al. reported findings in a cross-sectional study that recent antibiotic use correlated with significant reductions in bacterial diversity and increased *S. aureus* abundance [126]. In two prospective studies of antibiotics administered for AE-CRS, Merkley et al. and Liu et al. observed conflicting effects on bacterial diversity, where one study found increased diversity and the other study found decreased diversity after therapy [149, 150]. These studies examining the effects of medical therapy all contain very small cohorts, with variable follow-up periods, and concerns of acute exacerbations on top of the underlying chronic disorder. Although provocative, clearly further work using well-defined cohorts will be required to understand short-term, long-term, and individualized effects of medical therapies on the sinonasal microbiome.

Ramakrishnan and colleagues identified features of the sinus microbiota at the time of ESS that predict postsurgical outcomes [127]. Whereas baseline disease severity was not a predictor of outcome, higher biodiversity and increased levels of *Corynebacteria* predicted patients with favorable treatment outcomes, consistent with prior reports of *Corynebacteria* as "keystone" species with roles in pathogen exclusion. Major interventions for CRS, namely surgery and perioperative antibiotic administration, appear to result in dramatic shifts in microbiota, where some

patients maintain a new core microbiota and others return to their pretreatment features. The variable degree of resilience to therapy is not completely understood. Jain et al. reported 23 patients undergoing endoscopic sinus surgery and also observed unpredictable shifts in community composition with high inter-subject variability, but a general association with increased richness after intervention [151]. Kim et al. performed a prospective, randomized, single-blinded trial to evaluate the effects of minimally invasive balloon sinuplasty versus large antrostomy surgery on maxillary sinus microbiota and inflammation [152]. The authors found no difference in bacterial burden, cytokine profiles, or endoscopy scores between the two sides. However, significant differences in relative postoperative abundance of *Staphylococcus*, *Lactococcus*, and *Cyanobacteria* were noted between sides suggesting that the local anatomic environment may influence surface microbial colonization.

Microbiome Modulation Opportunities

Prebiotic or probiotic administration is of significant interest in CRS as an alternative method to the extensive antibiotic use associated with this disease. The goal of directing the microbiome away from pathogen colonization and dominance, toward restoration of diverse healthy commensal organisms, aligns with other parameters of successful disease management. Preclinical study suggests potential value of probiotic manipulation for CRS through direct immune modulation of PBMCs [153], and by antagonism of colonization by the sinus pathogen, *S. aureus* [154]. Clinical studies at this time are nascent, but have not found success.

A prospective, randomized, double-blind, placebo-controlled trial of an oral probiotic *Lactobacillus* strain was not found to have any effect on sinonasal quality of life in a study involving 77 CRS patients [155]. Several studies examining intranasal administration of probiotics have been recently reported. One study used a combination of *Streptococcus salivarius* and *Streptococcus oralis* applied intranasally for 1 week to demonstrate beneficial changes in nasal microbiota composition at 1 month posttreatment; however, clinical disease assessment was not undertaken [156]. Martensson and colleagues evaluated the effects of topical intranasal instillation of lactic acid bacteria on commensal microbiota and prevention of infections [157]. A mixture of 9 Lactobacilli and 4 Bifidobacteria species were applied in a single dose in 22 healthy subjects using a sham-controlled, double-blinded, crossover study design. No negative effects on the commensal bacteria were observed, and the authors reported no inflammatory response through assay of nasal lavage cytokines. Subsequent clinical evaluation using a similar study design in 20 CRSsNP patients, however, noted no effects in nasal symptoms, microbiology, or nasal lavage inflammatory cytokines when compared to sham control [158].

Rational selection of a probiotic in CRS, or CRS-subtypes, is lacking given the differences in existing microbiome studies, and limitations for speciation guiding strain selection. These issues could contribute to type II error in probiotic studies. Dosage, duration of treatment, durability of effects, and route of administration

(oral vs. topical intranasal) are unclear. Similarly, the concept of "mucus transplants" akin to fecal microbiota transplant (FMT) has been proposed, but to date there are no clinical or preclinical studies for this approach. Although exciting and innovative, little has been defined in microbiome modulation for CRS, and this type of therapy remains experimental in the clinical realm.

Conclusion

AR and CRS are highly prevalent and burdensome diseases, in which host–microbe interactions in the densely colonized upper airway are increasingly recognized to contribute to clinical and molecular disease hallmarks. Overabundance of pathogens, loss of commensal microorganisms, decreased microbial diversity, and microbial network instability of the sinus microbiome have all been demonstrated in numerous human studies. However, the beneficial/harmful functions of airway surface microbiota, their role in disease pathophysiology, mechanisms for host regulation of airway microbiota, and the capacity for restoration of disrupted microbiota ("dysbiosis") are key unresolved questions, primarily because human studies to date have utilized descriptive, cross-sectional study designs. Well-defined cohorts, longitudinal sampling, accounting for treatment-associated variables and confounding factors, and further attempts to move beyond associations towards causality are requisite steps to build on these studies.

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Chapter 5
The Role of the Microbiome in Asthma Inception and Phenotype

Steven R. White and Yvonne J. Huang

Introduction

Asthma continues to be one of the most common chronic diseases worldwide. It affects almost 300 million people and has an increasing prevalence in developed countries, including the U.S. As important as it is in childhood, where it affects up to 10% of children, it is also of major importance to the adult population. Many children with asthma will continue to have asthma their entire lives; others will experience some temporary abatement followed by recrudescence. Asthma that occurs de novo in adults, without any evidence of airway hyperreactivity or narrowing in childhood, is increasingly recognized. Indeed, asthma in the elderly has been recognized for decades, and the prevalence of asthma in this population also is increasing. The mechanisms proposed by which people develop asthma or are predisposed to its development are numerous. From a century ago when the origin of asthma was considered to be allergic in nature, we now understand increasingly that asthma is a rich interplay of genetics, inflammation, and environmental exposures. Of the latter, allergens, pollutants, and viral infection long have been identified, but increasingly, both the microbiome of the lungs and that of the gastrointestinal system ("gut microbiome") are increasingly understood to have some role, though the mechanisms by which each might do so, and the interplay between them, and of each with other environmental and inflammatory cues, are yet to be understood.

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This goals of this chapter are the following: (1) to synthesize recent evidence on the *airway microbiome* and its potential role in chronic asthma and asthma phenotypes; (2) to summarize evidence on the role of environmental microbial exposures and the developing upper airway and gut microbiomes in infancy on early-life asthma; and (3) to highlight the scientific challenges, critical research questions, and promising avenues to advance understanding of the microbiome's impact on asthma. Current knowledge on asthma biology and clinical characteristics also will be discussed, for the purpose of and tailored to these goals.

The Microbiome of the Airways in Asthma

Overview

There is now recognition gained over the past decade that the airways are not sterile in the normal state, and that indeed there exists an ecology of bacteria, likely together with fungi and viruses, in the airways. In this chapter the airway microbiome is defined as the composition of the bacterial and fungal organisms from trachea to alveoli as identified by an array of their genomes from next-generation sequencing. However, sampling the lower airways poses challenges (as discussed below). Studies focused on understanding the role of "airway" microbiota on asthma inception in early life predominantly have evaluated the upper respiratory tract (e.g. nasopharyngeal sampling) rather than the lower airways. Differences in the microbiome of the upper and lower respiratory tract both have been associated with asthma and are discussed separately in this chapter.

Studying the airway microbiome in asthma is motivated by the following important questions: First, do differences or changes in the microbiome have a role in modulating airway inflammation? Second, do the changes in airway ecology and the microbiome noted in asthma track with any of the defined asthma phenotypes in clinically important ways? Indeed, a dysbiotic airway microbiome may be correlated to or even essential to a select phenotype. Third, which comes first – a dysbiotic airway microbiome followed by airway inflammation, or airway inflammation (be it eosinophilic, neutrophilic, both or neither) that leads over time to altered ecological niches that support only select airway microbiota? And finally, if we manage through some manipulation (be it drugs, antibiotics, probiotics or something else) to nudge the airway ecology back towards a more "normal" state, will we in turn downregulate or modulate airway inflammation and perhaps clinical asthma control?

The relationships between the airway microbiome and asthma inflammatory phenotypes are complex. Defining the relationships between the airway microbiome and these phenotypes, however, might inform better our understanding of the underlying pathophysiology of each phenotype. Further, markers within the microbiome, be it the abundance of particular taxa, a diversity index, or ratio of select taxa, may serve as biomarkers that add to inflammatory, genetic, and clinical

biomarkers to define phenotypes and guide the use of therapies. The past decade has seen the utility of inflammatory biomarkers in the definition of phenotypes such as the usefulness of blood and sputum eosinophil counts as a marker for the type-2 (T2) high phenotype that predicts the response to therapies directed against interleukin (IL)-5 or the IL-4Rα receptor. Yet even here, patients who meet the criteria for this phenotype have, at best, a 50% response to these agents. The door is open to the potential use of the microbiome to shape and sharpen our understanding of asthma endotypes.

Further, a deeper understanding of the microbiome in chronic asthma may lead to therapies directed against select microbiota. As we will discuss later in this chapter, first steps have already been taken with the use of antibiotics such as azithromycin. Better and more targeted therapies, perhaps including probiotics (live microorganisms that are intended to have health benefits), prebiotics (nondigestible food components that selectively stimulate the growth or activity of desirable microorganisms) or bacterial products, may develop into adjunct therapies for select asthma phenotypes.

Defining the Airway Microbiome in Asthma

"The healthy lower airways are sterile" is a dogma taught over the last century of medicine. Indeed, the lungs were initially not considered important enough to study in the first phases of the Human Microbiome Project [1]. In retrospect, the flaw in such dogma is obvious: the lung is exposed to bacteria from early infancy from its continuous exposure to 8000 liters daily of inspired air and to secretions from the nose, oropharynx, and the gastrointestinal tract [2–4], and it would be remarkable indeed if the lung were not to be colonized by at least some microbiota in all that time. But the view of the sterility of the lung was ordered by the technology of its time: from the earliest days of microbiology, we could only consider those organisms that could be grown in culture, and it was uncommon indeed for any bacteria to be cultured from samples collected from the lung in its normal state. While organisms might be introduced to the lung from elsewhere, the innate defenses of the lung, including anti-bacterial defensive proteins and immune cells, and the "mucociliary elevator" that lift particles and contaminants including bacteria from peripheral airways to glottis, balanced this immigration exactly. Perturbations of this balance led to shorter-term infections such as bacterial, viral, or fungal pneumonia, and longer-term infections that characterize the progression of diseases such as cystic fibrosis [5–9]. But the mucociliary elevator and innate defense of the normal lung served to maintain sterility.

This dogma profoundly changed with the willingness of investigators to sample the lung more directly by bronchoscopy and by advent of next-generation sequencing. As described in earlier chapters, sequencing that exploited variations in the 16S rRNA gene, a highly conserved locus of the bacterial genome, permitted identification of nearly all bacterial organisms to an increasingly precisely identified level of

homology in a given ecological space, regardless of their ability to grow in any external culture system. Culture-independent profiling based on sequence polymorphisms in the 16S rRNA gene, and sequences unique to the fungal ribosome, permit identification of these microbiota and examination of the relationships of a microbial community to disease phenotypes [10]. Such a community would encompass both those organisms that were either transitory in passage down or back up the airways, or were more permanent survivors in a dedicated ecological niche. This community could be described by *taxonomy* (the identification and compositional abundance of microbes), the *diversity* of the microbial community both within each airway niche or between groups of patients (e.g. by asthma phenotype), and the *functionality* of these organisms based on readouts of their predicted gene functions or products. Such information provides insight into the interactions between microbiota in the same ecological space, and their ability to provoke (or not) the host-defense systems. Understanding the bacterial ecology of the airway then could be related to the changes within that airway specific to asthma: inflammation, changes in defensive mechanisms, and changes in airway structure.

How to Study the Airway Microbiome in Asthma

Before embarking on a review of the studies to date, one must consider how best to sample the airways, when such sampling should be done, and which patient populations should be studied. Chapters 1, 2 and 3 discussed study design and sampling considerations, including the methods for collection, processing, and subsequent data analysis (the bioinformatics "'pipeline"), with an eye towards enhancing reproducibility. This comes from hard-won experience in studies of other diseases and fields. We note the specific issues with sample collection with regard to asthma in this section.

The Problems of Sampling The first issue to be overcome is that of sampling: what is the best way to examine the airway microbiome in asthma? Differences in sampling methods, including the compartments sampled, may induce variability of results and both limit comparisons between studies and limit our ability to apply these findings to patient care.

The choice of which compartment to sample is the first question. Asthma is a disease of the large airways, and one would start with examining these airways – but *which* airways? The large airways can be seen as encompassing two regions: the trachea and main-stem bronchi, which are in direct communication with the oropharynx [11], and the lower-order conducting airways (generations 3 through about 10), which while connected to the trachea and to the peripheral airways may present as a unique microbial niche [12]. There are potential differences between these spaces, central, conducting, and peripheral, and each may be influenced by the microbiome of the adjoining space(s), the rates of microbial migration into and out of the space, elimination by any host defense factors, and microbial reproduction.

The next question is how to sample these spaces. Samples can be collected by bronchoscopy; an unprotected brush inserted through the channel of the scope will collect whatever material that has contaminated the channel to that point in time, including any oral and upper airway microbiota. A "protected" brush inside a catheter with a gelatin plug at its tip may fare better, but then special care is required to remove contaminants from around the protective sleeve. Brushing collects material, including epithelial cells and mucins, at the airway surface. The latter may have trapped (again, the "ciliary elevator") bacteria brought from the more peripheral airways. Thus, the endobronchial sample is an amalgam of what is there at that point in time, plus whatever has been lifted from below. An endobronchial washing with saline would invariably collect material from the peripheral airways unless a balloon is used to block this; this is cumbersome and requires significant expertise. Bronchial biopsies would include surface bacteria and also bacteria in the submucosa. In a comparison of paired endobronchial washes and biopsies obtained in a small cohort of patients with severe asthma, Millares et al. [13] found that the two types of samples had modestly different relative abundance, beta-diversity, and predicted functional capabilities based on an analytical method (PICRUSt). Finally, as one readily appreciates upon viewing data from any study using small brushes, there is a low biomass inherent both to the low absolute numbers of bacteria and the small sample mass both in the airway and on the brush. In sampling the central airways then, endobronchial brushings are generally used, but investigators must keep in mind these limitations.

What about the peripheral airways in asthma? Over the last two decades evidence has suggested the presence of small- and peripheral-airways disease in asthma [14–17]. Might then sampling of this space be worthwhile? This could be done by bronchoalveolar lavage (BAL); this fluid would represent all airways distal to the tip of a wedged bronchoscope, including the alveoli, and of course any organisms already in the channel of the bronchoscope. Such samples are then heterogeneous, and worse, of very low biomass, often an order of magnitude lower than that seen for the central airway samples in terms of bacterial burden [18].

The low biomass of either central or peripheral airway samples creates a significant additional technical burden, that of distinguishing what is in the lung from what is contaminating the sample (upper airway or from the scope channel) along with the background of any 16S rRNA that might be present in reagents and the various kits used for initial isolation [19, 20]. Both organizational (e.g., scope cleaning, testing of reagents) and computational methods can be used to ameliorate partially this problem [21] but investigations of the airway are challenged in a way that studies with much higher biomass, such as the GI (gut) microbiome, are not.

When to Study the Airway Microbiome Many of the adult studies done to date examine associations between clinical and biological parameters of asthma with the microbiome collected at a single time point. These cross-sectional studies are invaluable snapshots, particularly when compared to either normal subjects or to patients with other airways diseases such as COPD or cystic fibrosis. One recent review tabulates the findings of 31 cross-sectional studies done between 2010 and

early 2019 in pediatric and adult asthma; such tabulation clearly shows the substantial variance in key findings [22]. The major limitation of any cross-sectional study is, of course, the lack of data at a future point in time, and (usually) the inability to collect the same or similar data, particularly biological specimens, from previous points. Thus, cross-sectional studies are invaluable to delineate associations but less so to understand mechanisms.

A particular challenge for any asthma longitudinal study is the inability to collect samples of the lower airways repeatedly over time by bronchoscopy. While the risks of bronchoscopy are very low for asthma patients in a research setting [23, 24], relatively few research participants will consent to more than a single invasive procedure, and the costs associated with repeated bronchoscopy are prohibitive in any large trial. Serial sampling then might be best done via assessment of sputum. One early study demonstrated that the sputum microbiome differed in patients with severe or mild asthma compared to normal subjects [25]. But examination of these samples presupposes that the sputum microbiome is representative of that of the lower airway. Evidence that such a supposition is true (or true enough to be useful in investigation) comes from a direct comparison of sputum and lower airway microbiota in the AsthmaNet Microbiome study, a cooperative, multicenter observational trial that was sponsored by the National Heart Lung and Blood Institute (NHLBI) [26]. This study examined paired samples consisting of protected endobronchial brushings, induced sputum, oral washings, and nasal brushings in a cohort of patients with mild asthma. This study was careful to account for potential causes of contamination. As might be expected, although compositionally similar to the endobronchial microbiota of the lower airway, the microbiota in induced sputum were distinct and reflected enrichment of oral bacteria [26]. Patients with asthma or atopy were more likely to have bacterial taxonomy in induced sputum that reflected the lower airway and were more distinct from that seen in the oral cavity. This study suggests that within limits, repeated survey of the lower airway microbiome over time in patients with asthma (but not necessarily healthy subjects) may be approximated using induced sputum.

Considering Environmental Influences on Asthma and the Human Microbiome

Urbanization with attendant air and traffic pollution clearly are associated with increased asthma prevalence. While asthma prevalence has increased over the decades in highly developed countries, low- and middle-income countries now are seeing an increase, particularly among urban populations [27, 28]. The biologic, social, and environmental factors are complex and as yet incompletely understood but revolve around a rich interplay of air quality, pollution, diet, exercise or the lack of it, use of antibiotics, reduced exposures to "rural" allergens and increased exposures to city allergens, and changes in the patterns and types of childhood infection.

With these in mind, it is clear that the rural environment is associated with a lower prevalence of asthma [27, 29–31], a protection that is lost in rural to urban migration [32]. With the difficulties of quantifying exposures, lifestyle, and even whether a given residence is urban or rural, it is challenging to relate changes in the environment to changes in the microbiome that in turn might influence asthma.

One interesting approach to address the potential protective effect of the rural environment has been to examine the role of the farm microbiome and its microbial products on the susceptibility to asthma and atopy in children. Early-life farm exposures have been shown to reduce the risk of asthma in children [33–38]. In a study of 196 children with asthma with and without atopy, increased asthma severity was associated with an increased concentration of allergenic fungal species, high total fungal concentrations, and high bacterial richness in house dust [39, 40]. Differences in the prevalence of asthma and atopy are seen in children raised in different farming environments, such as between children of Amish (traditional farming) versus Hutterite (modern farming) heritage [38]. The farm environment can be carried indoors to mix with other influences including pet dander, food, dust mites, and other eukaryotic small organisms. Indoor microbiota are different in farm environments and are associated with asthma and atopy prevalence, and depending on context may be either protective or exacerbating [41, 42]. Here again, whether the indoor and farm microbiota elicit changes in the lower airway microbiome is not known. Indeed, in the study of Hrusch et al., that examined house dust differences in Amish versus Hutterite heritage, intranasal instillation of house dust extracts from either location to ovalbumin-sensitized and challenged mice elicited opposite effects: the house dust from Hutterite farms augmented whereas the Amish dust extracts inhibited the airway responsiveness, eosinophilia, and IgE levels induced by allergen [35]. But the differences in microbiota and other components in these two extracts were not defined, and so it is not clear what component of the dust is responsible. This one genre of work then illustrates some of the complexities of relating the larger environment to changes in the microbiome of a locale, and to determine whether a change in that microbiome, or its subsequent effect on the host, is responsible for asthma.

We often think of “the environment” as a macro or global or regional event, but in ecological terms the environment is everything about us from the great outdoors to our most personal indoor settings. For many, the indoor or “built” environment is key: most people in the developed world spend the majority of their time indoors in which the microbial community, both that within the home and that brought to the indoors, may be shared [43, 44]. Indeed, humans live within a personal, aerosol “bio-cloud” of their own microbiome that settles about them that can be used to identify individuals [45, 46], and in modern life it is straightforward to see that one’s aerosolized organisms (from oral pharynx, nose, skin but perhaps also exhaled from the lung) could be transferred to and then inhaled by another person. Indeed, this is a mechanism by which infection (bacterial or viral) may be transmitted. The proximity of people in the indoor environment, be it home, office, school, or day-care center, permits more efficient “sharing” and transfer of these microbiota. As one example, day care attendance early in life is associated with decreased asthma

prevalence at elementary school age and adolescence [47, 48], though there was no measurement or association of the environmental or child-carried microbiome in these studies.

Endotoxin exposure is a risk for wheezing but not for asthma [49, 50], an apparent contradiction that has not yet been resolved. Indeed, indoor microbial exposure to endotoxin and fungal antigens might reduce the risk of asthma in certain settings, as demonstrated in the *Prevention and Incidence of Asthma and Mite Allergy* birth cohort study of children with atopic mothers in New Zealand [51]. Counterbalancing these studies, both specific bacterial products (e.g., endotoxin) and fungal products (e.g., chitin, glucans) in indoor environments are associated with increased asthma prevalence in children [52–55]. Perhaps the organisms in the indoor environment themselves may change asthma susceptibility. One small study demonstrated that the microbiota in home dust differed in dwellings that housed low-income asthmatic children versus non-asthmatic children; in the former group the sampled house dust contained an increased abundance of Proteobacteria and Cyanobacteria [56]. One potentially important component of house dust can be pet dander, and it is clear that the indoor microbiota of homes with pets differ from homes that are pet-free [57–60]. Allergen presence and dog dander within a home are correlated with changes in the indoor microbiome in house dust [61]. Such differences in the indoor microbiome may in part account for the known association of dogs with protection from allergic sensitization and asthma in early life [62–64]. No studies to date have connected the taxonomy of house dust microbiota to that of the lower airway microbiome or has related house dust exposure to the changes in the airway microbiome in patients with asthma. Further, the specific biogeography within an indoor environment, ventilation, and the microbiomes of the occupants of a home all might contribute to the “bio-cloud” that we inhale, and thereby may modulate in some way our airway microbiome. Larger studies that associate these factors to risks of asthma, both in children and in adults, are needed.

The impact of seasonality in the larger outdoor environment (and perhaps also the indoor environment) on asthma has long been recognized in many regions of the world due to the variations in humidity, base temperature, and rapid changes in temperature, and high concentrations of air pollutants and allergens [65–75]. These are, of course, conditioned by individual clinical characteristics, phenotypes, and other causal and exacerbating factors. One potential mechanism among many by which these factors may elicit changes in asthma control may be by changing the airway microbiome. Studies that relate seasonality to the “bio-cloud” and thence to the lung microbiome clearly are needed.

The Airway Microbiome in Health

Having some understanding of the limitations of sampling and study design, and of where and how people with asthma might live, we can examine the airway microbiome in asthma. To do so, we first discuss briefly what is known about the “normal”

microbiome in health. One of the earliest papers to examine the normal lower airways community was that of Charlson et al. who sampled multiple sites in the pharynx, central airway by protected endobronchial brushing, and peripheral airways and alveoli by lavage, in six healthy subjects [11]. They demonstrated bacterial communities in the lower airways, both central and peripheral, that were low in biomass and that were indistinguishable from the pharyngeal flora. These bacteria were represented by five major phyla, including Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. Their data strongly suggested that the lower airway microbiome was a subset of that of the upper airway and occurred due to colonization from aspirated microorganisms. This work represented the first baseline data for the microbiome of lower healthy airways. The lungs, indeed, were not sterile. Subsequent studies that compare a disease-associated airway microbiome to normal generally have observed similar findings. For example, Morris et al. [76] examined the upper and lower airway microbiomes in 64 healthy subjects in a multisite study sponsored by the NHLBI *Lung HIV Microbiome Project* (LHMP); of these, 45 were nonsmokers and 19 were current smokers. Many of the microbiota identified in the lung were also noted in the mouth, but several, including *Haemophilus* and *Enterobacteriaceae*, were in higher proportion in the lower airways than upper, suggesting that the lung microbiome did not derive entirely from the mouth and pharynx. Further, while the upper microbiome differed between smoking and nonsmoking subjects, the lung microbiome did not. Another interesting observation was that while patients from eight different sites were included in their study, there were no significant differences in the diversity indices compared across the clinical centers, suggesting a remarkable uniformity in the healthy lung microbiome. Similar results with regard to the lack of difference in the lung microbiome between healthy nonsmokers and healthy smokers without lung disease were noted in bronchial wash samples [77] and bronchoalveolar lavage fluid [78]. Additional studies have examined the geography of the microbial communities of the pharynx (oral wash), nose (swab), stomach (gastric aspirate) and lung (BAL) in various combinations. One such study in 28 healthy subjects showed that both the oral and gastric microbiomes were both different and richer than that seen in the lower, peripheral airways [79]. Marked subject-to-subject variation was noted, a finding that has been seen in other studies of different microbiomes. Dickson et al. [12] examined 15 healthy subjects by bronchoscopy, sampling (in order) from the peripheral lung by BAL to conducting airways at several locations and then central airways by protected brushes. They demonstrated that spatial variation in microbiota within an individual was significantly less than variation across individuals, and that community richness decreased as samples went from trachea to conducting airway to peripheral airway. Another study of 86 normal subjects from the LHMP examined oral washes and bronchoalveolar lavage and demonstrated that the lower, peripheral airways as sampled by BAL did not mirror completely the oropharynx [80]. In a more recent study of 124 healthy subjects in which sputum was collected as part of a study examining the airway microbiome in patients with COPD, Firmicutes, Bacteroidetes, and Actinobacteria were the major phyla constituting 88% of the total reads in these healthy subjects; *Streptococcus*, *Veillonella,*

Prevotella, *Actinomyces*, and *Rothia* were the dominant genera [81]. The genus *Haemophilus*, in contrast, formed only 3% of the healthy microbiome. These findings were increasingly confirmed in subsequent studies comparing the normal versus asthmatic airway microbiomes in studies cited in the following sections. Similar findings have been reported in which a normal population is recruited as a control for COPD studies with reference to the lower airway microbiome (as examples, see [77, 78]).

It becomes clear then that the adult normal airways have a bacterial microbiome that is small in biomass, well defined, and derived in part, but only in part, from that of the upper airway. The major phyla that are reproducibly found include Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria. While there can be significant variation between normal, healthy subjects, there is less (but not zero) variation between regions of the lung. How is it different in individuals with asthma, *when* does it become different, and might a dysbiotic microbiome have a role in asthma and airway inflammation? In the next section we discuss first the evidence linking early-life exposures and the developing microbiomes of the upper airways as well as of the gut, to asthma development in childhood.

Early-Life Asthma and the Microbiome

Extensive evidence has highlighted the concept of a critical window in early life during which the developing immune system is shaped by exposures that influence subsequent risk for allergic diseases including asthma [82]. Such exposures can be categorized broadly as follows: (1) characteristics of the *external environment* that impact opportunity for microbial contact (e.g. farm vs. nonfarm household); (2) *lifestyle practices* that shape the establishment of microbiota and maturation of the microbiome (e.g. breastfeeding, pet ownership); (3) *medical events and treatments* that may support or interrupt microbiota establishment, its ecological succession and interactions with host immunity (e.g. respiratory virus infections, antibiotics, probiotics). Numerous studies have been published on these areas over the last several decades. Reported links to various types of exposures, coupled with mechanistic studies implicating the role of microbiota, have underscored the multifactorial nature of early-life interactions that shape risk for asthma. This section will highlight select studies that have contributed to this evidence. These include recent investigations that have applied advanced analytical methods to examine the microbiome coupled, in some cases, with mechanism-directed experiments. In doing so, such studies demonstrate the power of combining cross-disciplinary approaches to advance insights into the complex host–microbiota interactions that shape asthma pathogenesis.

The Early Microbiome in Asthma and the External Environment As mentioned earlier, differences in the prevalence of allergic conditions between persons living in different environments or geographic regions have long been recognized, well

before the current era of next-generation sequencing methods to survey microbial content. Epidemiologic comparisons between different populations of similar genetic ancestry have reported significant differences in the rates of allergy and asthma, observations that persist in more recent reports [35, 36, 38, 83, 84]. These include studies comparing individuals living in the Karelia region of Finland versus Russia [84], in farming versus nonfarming households of Germany and other parts of Europe [36, 83], and between Amish and Hutterite communities in North America [38, 85]. These and other similar data [86] support the now well-accepted notion that characteristics of the home environment can affect risk for childhood allergy or asthma.

Differences in hygiene levels and related living practices impact the frequency and nature of contact with microbes. Thus, many studies have examined markers of microbial load or the types of microbes found in household dust samples [36, 55, 87], finding differences associated with atopy or asthma. In general, a higher microbial load or diversity of microbes found in household samples is associated with a lower risk or prevalence of atopy or asthma. However, the specific microbes associated with these outcomes vary by study and location. This highlights not only geographical differences, but also the likely importance of the sum effects of a microbial community on microbiome–host interactions. A number of factors affect microbial content within homes, which include pets that are exposed to the outdoors and the flow or tracking of outside air or soil into homes [61, 64, 87]. As noted in the section concerning the indoor environment and the microbiome, recent studies also have shown interactions between household bacterial and allergen load that significantly modify their associations with asthma [88, 89], adding further complexity to how environmental exposures shape immune responses that lead to asthma.

To apply this knowledge in a way that may guide environmental interventions, a recent study aimed to quantify the "farm home microbiota" effect by developing an index ("FaRMI") derived from the relative abundances of bacteria/archaea measured in farm-home floor dust [90]. Relative abundance data from samples collected in a rural Finnish birth cohort were used to derive the index, which was then applied to samples from a nonfarm/suburban Finnish birth cohort. The overall microbial composition of samples was distinct between the two cohorts. Compared to suburban homes, rural home dust was characterized by higher bacterial richness and enriched in members of specific bacterial orders (Bacteroidales, Clostridiales, and Lactobacillales). No differences in fungal richness were identified. Among suburban homes a higher FaRMI in dust samples was negatively associated with asthma. Similar relationships were observed when FaRMI was derived and used to analyze samples from an independent German birth cohort. Moreover, the asthma-protective effect observed with a high FaRMI was independent of atopic sensitization, suggestive of microbiota-specific contribution. Further research is still needed to understand how environmental exposure to particular consortia of bacteria elicit immune-protective effects against asthma in early life.

Other recent studies have shed insight into this by phenotyping innate and adaptive immune responses between groups at differential risk for asthma, such as

among Amish versus Hutterite children. The prevalence of asthma and atopy is strikingly lower among the Amish, and significantly higher levels of endotoxin were measured in Amish house dust [38, 85]. Major differences also have been observed in the proportions and functional markers of innate immune cells in blood and in T-cell phenotypes [35]. As mentioned earlier from this study, using a mouse model of allergic asthma, intranasal administration of Amish house dust extracts prevented airway hyperreactivity and lung eosinophilia, contrasting from the effects observed with Hutterite house dust extract [38]. The protective effects were abrogated in mice deficient for MyD88 and Trif, two important molecules at intersecting innate immune-signaling pathways. Although no information is currently available on whether there are differences in the dust-associated microbial or allergen content, results from the in vivo models indicate that specific components in the Amish house dust are at least partly responsible for the observed protection against allergic airway inflammation. Similar observations were made in another study in which dust from dog-owning homes was administered to mice using models of asthma; this resulted in attenuation of allergic airway inflammation, with *Lactobacillus johnsonii* identified as one responsible species [91].

The GI Microbiome and Early-Life Asthma The establishment of gut microbiota is essential for normal immune development and discussion of this is important as it pertains to asthma risk in childhood. The gut microbiome is established in the same way the lung microbiome is starting shortly after birth, by aspiration or swallowing of oral microorganisms. Transfer of bacteria from the gut to the lung also may be indirect, as GI bacteria may be taken up into macrophages and dendritic cells that then may migrate to the lung [92]. The consequences of lacking microbiota, as seen in germ-free mice who are born and raised under sterile conditions, include underdeveloped gut mucosa-associated lymphoid tissue and dysfunctional immune responses [93]. That the gut microbiome plays a key role in the pathogenesis of early-onset asthma is supported also by the evidence that certain interventions, which affect maturation of the gut microbiome, are associated with atopy and asthma in pre-school and school-age children [94]. These include perinatal factors such as mode of delivery (Caesarean section) and exposure to antibiotics, while breastfeeding is associated with decreased risk [95–97]. These factors have been shown to affect the trajectory of gut microbiota development over the first two years of life [98]. Bokulich et al. [98] found that Cesarean section led to depleted Bacteroidetes populations in infants, altering establishment by maternal-derived bacteria that otherwise would occur via vaginal delivery. Antibiotic administration significantly suppressed Clostridiales, including *Lachnospiraceae*, which include species that produce butyrate and other short-chain fatty acids that regulate host immunity. As alluded to, the mechanisms through which gut commensal bacteria shape local and peripheral immune responses involve a multitude of pathways [99, 100]. The role of vitamin D in immune function [101] and the effects of short-chain fatty acids (SCFA) [97, 102] and biogenic amines, both of which are produced by certain gut bacteria [103–105], all may play a role in asthma pathogenesis.

A number of recent studies from large birth cohorts have investigated more specifically the relationships between infant gut microbiota composition and markers of atopy, asthma incidence or prevalence in later childhood [106–110]. Earlier culture-based studies provided the initial evidence that fecal prevalence of specific bacterial species differed between children who did or did not go on to develop atopy. For example, Kalliomaki et al. [111] observed a reduced ratio of *Bifidobacteria* to *Clostridia* isolated by culture, and in parallel found an overall difference in stool bacterial fatty acid profiles at age 3 weeks between infants who had evidence of atopy versus those who did not at age 12 months. Such differences observed from samples collected in very early life (within the first 1–3 months) may seem surprising, but similar observations have been made from other birth cohorts. For example, in a study of 319 infants enrolled in the Canadian Healthy Infant Longitudinal Development (CHILD) Study, Arrieta et al. [112] performed 16S rRNA gene sequencing of fecal samples collected at age 3 months and found decreased fecal prevalence of four bacterial genera members (*Lachnospira, Veillonella, Faecalibacterium, Rothia*) in children who were at increased risk for asthma. Infants at increased risk also had reduced fecal LPS concentration, as suggested by in silico analysis of predicted bacterial gene functions, and also reduced fecal levels of the SCFA acetate. Intriguingly, inoculation of germ-free mice with primary isolates from these four bacterial taxa ameliorated airway inflammation in adult progeny, demonstrating a causal role for members of these bacterial genera in allergic airway disease.

The exact "critical window" of microbiota–host interactions that sets a risk trajectory towards early atopic asthma remains to be firmly established. Other birth cohort studies have examined the gut microbiome at later time points (up to 1 year), finding relationships to subsequent asthma or atopy risk even in samples collected later in the first year of life [109, 110]. Among 690 infants in the Copenhagen Birth Cohort [110] (fecal samples analyzed at ages one week, one month and one year), the 1-year samples were compositionally distinct from the early time points. Yet, of the two clusters defining microbiota differences in the 1-year samples, this distinction was most apparent among infants born to an asthmatic mother. Moreover, it was within this group of at-risk infants (i.e. asthmatic mothers) that associations with asthma at age 5 were characterized by differences in the relative abundance of *Veillonella, Lachnospiraceae incertae sedis, Bifidobacterium, Alistipes and Ruminococcus*, and other bacteria. No significant links to later asthma with the 1-year gut microbiota composition were observed among the infants born to non-asthmatic mothers. The noted interaction between gut microbial community "type" and a family history of asthma highlights the intersecting factors that add complexity to understanding mechanisms that result in increased asthma risk.

Beyond bacteria, the role of fungal communities (also referred to as mycobiota) is of tremendous interest. However, human investigations focusing on this and in the context of pediatric asthma remain sparse. Given the dynamics of the gut microbiome in early life, along with established knowledge regarding immune responses to fungi, it is reasonable to suspect fungi play a role in asthma. Direct interactions between fungi and bacteria also may be important, but much remains unknown

about inter-kingdom interactions and their role in human diseases. However, data from mouse models have shown, broadly, that fungi play a role in type 2 inflammatory responses. For example, oral treatment of mice with antifungal drugs results in restructuring of their gut mycobiota (reduced *Candida*, increased *Aspergillus* and other fungal species) and in a mouse model of asthma, led to increased allergic lung inflammation characterized by eosinophil infiltration, and increased type 2 immune responses measured in blood [113]. Mice whose intestinal tracts were newly colonized with *Candida albicans* displayed not only fungal-specific Th17 responses, but also increased susceptibility to allergic airway inflammation [114]. Studies from human birth cohorts have reported differences in the relative abundances of specific fungi as a feature of atopy- and asthma-associated gut dysbiosis [109, 112]. In a U.S. cohort [109] clustering of bacterial and fungal community data revealed a cluster characterized by low relative abundance of *Bifidobacteria*, *Akkermansia*, and *Faecalibacterium* and a higher relative abundance of *Candida* and *Rhodotorula* fungi. Infants in this cluster had the highest risk of atopic sensitization to aeroallergens at age 2.

The Nasopharyngeal Microbiome and Early-Life Asthma To date, much of the literature studying the role of the microbiome in childhood asthma has focused on the environment and trajectories of gut microbiota establishment and succession. There has been recent interest as well in the upper respiratory tract (URT) microbiome, in particular the nasopharyngeal (NP) compartment. Several types of sampling approaches have been applied to collect samples from the URT, including nasal swab, nasal aspirate, and hypopharyngeal swabs via the oral cavity [115–121]. Despite differences in sample collection, studies utilizing any of these specimen types have identified links between URT bacterial microbiota and the development of childhood asthma. Like the gut, the composition of nasopharyngeal bacteria is dynamic in the first few weeks to months of life, even in healthy infants [115, 117]. Similar dynamism has been described for bacteria profiled from hypopharyngeal aspirate samples taken in the first 3 months of life [116].

In a study of 112 infants sampled frequently in the first year of life, factors differentially associated with NP bacterial composition included mode of delivery, infant feeding, crowding, and recent antibiotic use [115]. In contrast to the lower respiratory tract, *Corynebacterium* and *Dolosigranulum* are more prevalent members of the NP microbiome, and evidence suggests they are associated with healthy states. Bosch et al. [115] observed that children experiencing more respiratory tract infections (RTIs) in the first year of life already displayed an aberrant microbiota developmental trajectory at age one month, compared to children experiencing fewer or no RTIs. The observed alterations involved decreased stability of the NP microbial community over time, reduction in *Corynebacterium* and *Dolosigranulum*, and early enrichment in *Moraxella*.

Viruses as a cause of RTIs (e.g. rhinovirus and respiratory syncytial virus; RV and RSV) are an important risk factor for childhood asthma [122]. Thus, studies examining longitudinal relationships between viral RTIs, changes in URT

microbiota (nasal or hypopharyngeal) and asthma outcomes, are of great interest to elucidate the potential role that airway bacteria may play in modulating asthma risk. In a multicenter cohort of infants hospitalized with RSV-induced bronchiolitis, delayed clearance of RSV (defined by the same RSV subtype identified three weeks later) was associated with a *Haemophilus*-dominant NP microbiome at the time of initial hospitalization [119]. This suggests that an individual's existing NP microbiota pattern may play a role in determining the severity or outcome of viral RTIs. In a study of 234 infants from an Australian birth cohort, Teo et al. observed that NP bacterial profiles defined by predominance of *Moraxella, Streptococcus, Haemophilus* were significantly associated with acute viral RTIs [121]. Intriguingly, shifts in NP bacterial composition were detected in samples obtained preceding RTIs. Moreover, the consequences of having an RTI-associated NP bacterial profile (i.e. defined by *Moraxella, Streptococcus or Haemophilus*) differed by atopic status. Atopic children were more likely to have a "persistent wheeze" phenotype by age 5 in contrast to non-atopic children with the same NP microbiota profile. These observations suggest that allergic state modifies the outcome of viral RTIs coupled to altered NP bacterial composition.

Mechanistic links between dysbiosis of the upper airway microbiome and childhood asthma are not fully understood. In the COPSAC birth cohort, higher relative abundances of *Veillonella* and *Prevotella* in hypopharyngeal aspirates collected at age one month were associated with asthma by age 6 years and associated with reduced TNF-α and IL-1β and increased CCL2 and CCL17 in nasal epithelial lining fluid, markers of both Type 1 and Type 2-related immune responses [123]. Another recent study from this cohort observed associations between airway bacterial richness at age 1 week and allergic rhinitis at age 6 years, which was mediated by an epigenetic signature correlating with expression of genes for lysosome and bacterial invasion of epithelial cell pathways [124]. More research is needed to dissect causal relationships between altered airway microbiota, acute viral RTIs, and the associated immune responses to understand how these factors intersect and temporally influence asthma risk.

Lastly, whether differences in the NP microbiome may modulate asthma outcomes in older children has been examined in several recent studies. Zhou et al. analyzed nasal blow samples from 214 children (mean age 8 years) to determine if bacterial composition changed at the onset of loss of asthma control and whether particular microbiota characteristics associated with the number of these events over the course of one year [120]. Children whose nasal microbiota was dominated by *Corynebacterium* and *Dolosigranulum* experienced the lowest number of events. Furthermore, shifts to a *Moraxella*-dominated nasal microbiota detected at the onset of the event was associated with greater likelihood of progressing to a severe asthma exacerbation. Similar observations related to *Moraxella*-dominant nasal microbiota and risk of exacerbations were seen in a study of 413 children between the ages of 6 and 17 years [125]. Lung function and bronchial hyper-reactivity have also been associated with greater NP relative abundance of *Streptococcus* and *Staphylococcus*, respectively, among older children with asthma [118].

The Microbiome in Adult Asthma

Exploration of the lower airway microbiome in adults with asthma follows from the studies outlined above in early childhood asthma. Organisms introduced early in life might provoke inflammation and injury, or limit the responses to that inflammation, such that over time repeated microbial exposure or periodic insult could lead to an on-going inflammatory state that would be locked-in by early adult life. This *dysbiotic theory* could include not just bacteria but also viral infection, and could explain how repeated infection, overt or sub-clinical, would serve as an *effector* that over time could lead stimulate worsening inflammation and airway damage in adult asthma. This could be combined with the insults from other environmental stimuli such as allergens and pollutants, and indeed such multiple stimuli would work in concert or synergistically. Alternately, inflammation and injury from these other sources could over time alter the ecological space in the lower airway in a way that ordinary, commensal organisms seen in very low biomass could no longer survive, to be replaced (or augmented and supplemented) by new phyla and genera. This *lung disease theory* would suggest that for the most part, the microbiome was more of a reactor or even an innocent bystander, being acted on rather than acting to change the airway micro-environment. Of course, both mechanisms could be operative as a *synergistic theory* of on-going and mutually reinforcing airway injury, such that the microbiome is both effector and reactor. Finally, there are clearly a certain percentage of adults who lack any evidence of asthma in childhood, who lack atopy, who nevertheless have asthma [126–128]. Asthma in these patients is clearly heterogenous in nature and due to several phenotypes [128, 129]. A de novo dysbiosis of the lower airway microbiome might explain how these patients developed airway inflammation and clinical symptoms, and as in children, it is also possible that the microbiome might be a reactor or bystander. With this in mind, over the past decade there have been a number of studies that have examined the association of the airway microbiome and asthma in adults.

One early study used endobronchial brushes in a small cohort of subjects to demonstrate an increased relative abundance of Proteobacteria, particularly *Haemophilus*, and a decreased abundance of *Prevotella* in adult patients with either asthma or COPD compared to control subjects [130]. Millares et al. [13] examined 13 patients with severe asthma; this study did not include a control cohort, but did demonstrate a significant abundance of *Streptococcus* and *Prevotella* in bronchial biopsies. Goleva et al. [131] demonstrated that the microbiome present in bronchoalveolar lavage fluid of control subjects and subjects with either corticosteroid “resistant” or “sensitive” asthma differed modestly in relative abundance of selected genera, though there were no differences at the phylum level between asthmatic patients and normal subjects. Further, the overall bacterial burden was low. Another small study demonstrated in a cohort of 10 control subjects and 10 subjects with mild asthma that three major phyla, Firmicutes, Actinobacteria, and Proteobacteria, accounted for over 90% of total 16S rRNA sequences profiled from the sputum supernatants of subjects with mild asthma. Here again, Proteobacteria were significantly enriched

compared to microbial communities in the sputum of control subjects [132]. These data suggested that the lower airway microbiome could indeed differ in asthma. However, differences in sample collection and sample location within the lung, varying phenotypes of asthmatic subjects, and differing use of medications, particularly inhaled and oral corticosteroids, were likely significant confounders.

Larger studies in time confirmed first that the airway microbiome of patients with asthma were different, if modestly so, from that of the normal airway microbiome, and began to address the more obvious confounders. Using brush samples previously collected in the Macrolides in Asthma (MIA) study, Huang et al. [133] demonstrated higher 16S rRNA amplicon concentrations and diversity in endobronchial brushings obtained from asthmatic patients versus healthy controls that correlated with bronchial hyperresponsiveness. This study was large, 65 adults with sub-optimally controlled, mild to moderate asthma, and contained a small control cohort. Many but not all subjects had evidence of 16S rRNA in their endobronchial brushes, and not all of these could be amplified. Of the 42 asthmatic and 5 control subjects with sufficient product, a clear difference in bacterial burden could be identified. While the normal airway was not sterile, the asthmatic airway had a greater bacterial burden. Going beyond a taxonomic approach, the study demonstrated that the degree of bronchial hyperreactivity to methacholine correlated not to the relative abundance of any genus or phylum, but rather to the overall community diversity of the bacterial population. This approach emphasized that the overall ecological community perhaps matters more than simply an "over" or "under" abundance of a single bacteria. This work was among the first to demonstrate a relation of bacterial burden and community diversity to a physiologic parameter important to asthma.

A follow-on study then examined patients with more severe asthma, collected from the *Bronchoscopic Exploratory Research Study of Biomarkers in Corticosteroid-refractory Asthma* (BOBCAT) study [134]. The ability to examine larger populations was facilitated by the ability to "tag on" to previous sample collections, illustrating the importance of biospecimen collections in asthma studies, while also imposing limitations as to how samples may have been collected, processed and stored. In this study, patients with severe asthma had differences in bacterial composition of endobronchial brushes based on body mass index, assessment in asthma symptom control, the number of sputum neutrophils, and the number of eosinophils in bronchial biopsies. These bacterial communities did not diverge from normal in the same way: for example, microbial communities associated with poor symptom control and sputum neutrophils were predominant for Proteobacteria, whereas patients with a higher body mass index had an enrichment in airway microbiota for Bacteroidetes and Firmicutes. While it was difficult with a smaller cohort to examine differences in phenotypes, expression of several Th-17 genes in airway epithelial cells was associated with Proteobacteria dominance in the microbiome. Finally, the airway dysbiosis in patients with severe asthma appeared to differ from that noted in subjects with milder asthma who were using inhaled corticosteroids.

Other asthma cohorts also focused on relating asthma clinical parameters to the microbiome. Denner et al. [18] related the abundance of select taxa to corticosteroid use and to pulmonary function, specifically FEV1. Using endobronchial brushings,

they found that *Lactobacillus*, *Pseudomonas*, and *Rickettsia* were significantly enriched in samples from asthmatic patients, whereas *Prevotella*, *Streptococcus*, and *Veillonella* were enriched in brush samples from control subjects. In this regard their control data agreed with that of Charlson and other studies with a normal cohort, increasing our confidence that such organisms may be considered, in at least most healthy people, as commensal. Further, Denner et al. found that *Pseudomonas* was in greater abundance in patients receiving oral corticosteroids and with a lower FEV1 – that is to say, in patients with more severe asthma. Zhang et al. [25] using induced sputum also were able to demonstrate an increased prevalence of *Pseudomonas* in patients with severe asthma compared to controls. Li et al. [135] demonstrated a higher abundance of *Pseudomonadaceae* in severe asthmatic subjects compared to those with milder disease and with control subjects. Other studies using sputum or brushes have not replicated the increased relative abundance of *Pseudomonas* but have instead observed enrichment of genera such as *Neisseria* and *Moraxella* [130, 132, 136, 137], illustrating the importance of method, sample collection, and patient population. Further, not every study finds a difference between healthy subjects and patients with asthma, particularly if the disease is mild enough that patients are not being treated with inhaled corticosteroids [138]. Indeed, in studies in which patients with differing disease severity are included, those with mild disease generally have few differences from normal [18, 137], though one recent paper has demonstrated association of both sputum and oral microbiota to immunologic features such as atopy status and the presence/absence of a T2 phenotype in subjects with mild asthma [136]. It is clear that patients with more severe asthma, particularly those with frequent exacerbations or requiring the use of high-dose inhaled or oral corticosteroids, have a more disordered airway microbiome.

Among the ecological markers of a microbial community is the diversity of the community, both within a defined group of subjects (alpha-diversity) and between groups (beta-diversity). In many chronic illnesses in which the microbiome has been examined (as one example, Crohn's disease [139]), alpha-diversity is decreased in patients with illness compared to control – that is to say, there are fewer different types of bacteria in the ecological space, reflecting an ecological collapse that may be related to the disease state and the consequences of the two-way response of host and microbial community. Studies to date in asthma have been mixed with regard to changes in diversity. Two fairly early studies demonstrated a higher diversity in asthmatic patients compared to healthy controls [132, 133], whereas other studies found no significant changes between these groups [135, 138, 140]. The Denner study noted decreased diversity in asthma patients versus healthy subjects as measured by the Shannon alpha-diversity index [18], while the AsthmaNet microbiome study found that diversity as measured by Faith's phylogenetic diversity index, a phylogeny-based measure of biodiversity, was *increased* in asthmatic patients versus healthy subjects [137]. Other recent studies have demonstrated lower alpha-diversity indices in patients with severe asthma versus milder disease; these studies have been larger and have had a greater proportion of asthma patients with severe disease [18, 26, 141]. The weight of the evidence currently suggests that in asthma, as in other chronic illness, microbial diversity decreases, though this may be

difficult to demonstrate in patients with mild airways disease. Moreover, there are many ways of examining and comparing microbial diversity, and the inconsistencies between the above noted studies may reflect this. These measures represent today our best efforts to understand what shapes differences between groups of patients.

Longitudinal Studies The study of the airway microbiome in adult asthma has been limited by the lack of longitudinal studies. Several studies have been done in children with asthma via sampling of the nasopharynx, which as previously noted may not reflect the lower airways. These studies have identified links between URT bacterial microbiota and the subsequent development of childhood asthma [115–121]. Similar studies have not been done in adults to date; we therefore do not know the time-associated changes in the asthmatic microbiome. Indeed, we do not even know the dynamic changes that occur in the normal lower airway microbiome and how these might be influenced by the environment and diet. It is well established that the GI microbiome is highly variable in early life and is great influenced on a daily basis by diet, environment, and the use of antibiotics [142–145]. Tantalizing clues summarized in following sections suggest that the environment and antibiotics may alter the airway microbiome as well, and that management of such alterations might well improve asthma control. To date, however, the longitudinal human studies, greatly needed, are lacking.

In summary, the airway adult microbiome is different in patients with asthma. After consideration of all the differences in study methods, sampling locations, sequencing methods, and varying patient populations and disease severity, both the taxonomy and diversity of the asthmatic adult microbiome differs somewhat from health and changes more as disease state worsens. What we cannot demonstrate yet is a distinct profile that uniquely and near-irrevocably makes clear that, given this microbiome, this patient must have asthma. Simply put: there are no differences in kind, only in degree.

The GI Microbiome in Adult Asthma As previously discussed, the GI microbiome clearly has a modulatory and very likely contributory role in the development of early-life, childhood asthma. It is becoming clearer that the GI microbiome may also have an on-going role in adult asthma, by introduction of organisms to the lung via aspiration, by production of immunomodulatory factors, by alterations in the function of immune cells, all of which then lead to changes in airway inflammation. The relationships between the GI microbiota, their products and immunomodulatory signals, and the lung (i.e. the *gut-lung axis*) could extend, regulate and exacerbate T2-high responses in asthma, and may also have a role in Th17-driven asthma [146, 147]. Metabolites from GI microbiota also could influence T cell plasticity and function and dendritic cell function.

Current estimates suggest that the adult gut contains approximately 10^{14} bacteria, perhaps 7 to 8 log-fold more than the lung; two-thirds of these are specific to an individual [148, 149]. Four phyla, Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria predominate in the GI microbiome as in the lung. Genera such as

Clostridium, *Faecalibacterium*, *Ruminococcus*, *Roseburia*, *Eubacterium*, *Bifidobacterium*, *Prevotella*, and *Bacteroides* are dominant in the normal intestinal microbiome [149]; many of these are found in low abundance if at all in the normal lung microbiome. Under normal circumstances the predominant GI bacteria both prevent the growth and aggression of harmful and pathogenic microbiota and participate in a number of beneficial immune modulating functions [149, 150]. As one example, they aid in digesting food products by fermenting complex carbohydrates that then produces SCFA that regulate inflammation and allergic responses [151–153]. A dysbiotic GI microbiome with a disrupted ecology not only leads to intestinal inflammation and dysfunction but also to worsened allergic inflammation and responses elsewhere [151]. A number of factors, including age, diet and fiber, childbirth, antibiotic ingestion, and intestinal disease all can lead to a GI microbiome that is temporarily or permanently dysbiotic [149, 151].

Our understanding of the relative contributions of the GI and lung microbiomes in adult asthma is evolving. The *gut-lung axis* is best considered as a transfer of metabolites, immune cells and immunomodulatory signalers from the gut to the lung (though reverse transfer could also occur), such that changes in gut microbial ecology or a gut dysbiosis could influence adult respiratory diseases including asthma [154–156]. A recent review examines the potential immune regulators that may be generated by a microbiome [153]. Many studies that examine this axis focus on acute infection models (e.g., influenza, pneumonia, mycobacteria) [152, 157–160], and the literature regarding early-life interactions was summarized above. Both the GI and lung microbiomes may be modulated by allergens (inhaled and/or swallowed) that both directly alter the respective barrier function in each system and elicit immune cell activation. House dust mite antigen, long known to be an allergic irritant in the lungs and indeed used in mouse models of T2-mediated allergic lung inflammation, also impairs the barrier function of the intestine upon ingestion [161]; likewise, particulate matter found in air pollution not only can elicit airway inflammation but also colonic epithelial inflammation [162]. A "leaky" barrier may allow ingress of bacteria; this has been demonstrated in clinical situations such as the adult respiratory distress syndrome [163] but such transfer has not been demonstrated in chronic inflammatory lung diseases. Intestinal epithelial cells and immune cells may assimilate signals directly from the directly abutting or nearby GI microbiome in ways that both shape a local response and a response at distal sites, including the lung [164]. As one example relevant to asthma, certain *Bacteroides* species that can synthesize polysaccharide A (PSA), introduced into germ-free mice, elicit a higher number of circulating IL-10 producing CD4+ T cells and Th1 cells compared to non-PSA synthesizing species [165]; this might drive, for example, T2-low asthma. In a mouse model of allergic airways disease that results from administration of antibiotics and disruption of the GI microbiome followed by ovalbumin sensitization and challenge, introduction of *Candida albicans* to the gut elicits a greater Th2-mediated inflammatory airway response [166]. In a study of young and old mice that mimics the effect of aging, older mice challenged with house dust mite allergen had greater airway inflammation, and had a different GI microbial structure with a decrease in the ratio of Bacteroidetes to Firmicutes, compared to

similarly-challenged young mice [167]. The combination of GI microbiome manipulations and allergen airway challenge, or lung infection challenge, in animal models is a particularly useful model that may allow us to understand the interplay of the gut-lung axis on airway inflammation, particularly in longitudinal models.

Studies of the gut-lung axis in adults humans with asthma are few to date. A recent interesting pilot study has demonstrated differences in the gut bacterial community structure in a group of adults with mild-to-moderate asthma compared to control subjects without known lung disease [168]. The gut microbiome within each subject was stable in the absence of changes in asthma status, while there was a strong association between FEV1 and differences in bacterial composition at the phylum level with changes in both Bacteroidetes and Firmicutes and a lower B/F ratio in asthmatic subjects. Future studies will need to examine both gut microbial parameters and circulating mediators that may be secreted by GI immune cells provoked by the gut microbiome, and then sample (by bronchoscopy if possible) changes in local lung inflammation, particularly T cell phenotypes and the presence of cytokines released by different T-helper cell populations. These more mechanistic clinical studies then may help delineate the role of the gut-lung axis in asthma.

Asthma Phenotypes and the Airway Microbiome

Of particular importance among adult asthma microbiome studies done to date have been those relating the microbiome to biological markers of a particular phenotype.

Key Asthma Phenotypes and Endotypes A complete review of the state of knowledge of asthma phenotypes is beyond the scope of this chapter, and readers are referred to excellent, recent reviews of this topic [169–171]. Heterogeneity in asthma and airway inflammation was understood over a century ago [172], and it is clear today that asthma is a heterogenous and complex disease with genetic, environmental, immunological, and behavioral inputs that cannot be explained by one single pathophysiologic mechanism. Over the past two decades, our understanding of asthma has been guided by the T2-inflammation hypothesis that provides an organizing immunologic and molecular framework for the fundamental and well-known associations of atopy, early life exposures, and eosinophilic airway inflammation. Both CD4+ helper T lymphocytes (Th2) and innate lymphoid cells (ILC2) [173] generated as a response to type-2 inflammation release signaling cytokines (such as IL-4, IL-5, and IL-13) and chemokines that drive a particular type of airway inflammation notable for eosinophilic infiltration of the mucosa and submucosa [174, 175]. This idea is further refined by the recognition of the endotype, that is, a condition or phenotype that is defined by a distinct functional or pathobiological mechanism [176], that might be defined (at least somewhat) by statistical clustering or big-data approaches, the use of molecular or genetic signatures, and the response to biological therapies, showing an end-result, real-world usefulness of the defined endotype [177]. The T2-high endotype, driven by eosinophilic inflamma-

tion that is signaled by T2-related cells that are in turn induced by epithelial cell, viral, and allergic/atopic stimulation [178], and is responsive to anti-IL-4 and anti-IL-5 therapies, is perhaps the best understood of the asthma syndromes and accounts for perhaps one-fourth of all patients with asthma. The T2-low phenotypes, best understood as “not T2-high”, clearly are heterogenous, varied, and not well separated from each other. A neutrophil-dominant asthma phenotype characterized by high proportion of sputum neutrophils, perhaps driven by cytokines such as IL-17 and IL-22, and mixed neutrophil-eosinophil phenotypes, are part of this T2-low paradigm [179–183]. Clearly microbial (bacterial or fungal) products, or the organisms themselves, could be part of the signaling processes in either T2-high or T2-low inflammation.

The T2 Phenotypes and the Microbiome Given the state of asthma phenotypes today, the incorporation of key microbial markers might help improve our ability to define and apply these phenotypes to patient care. Studies to date have attempted to examine certain phenotype markers, generally dividing their cohort based on one or a couple of key clinical or biomarkers, and then describing differences in the microbiome. Again, these are generally cross-sectional studies with small cohorts compared to the typical sizes of phenotype-driven biological drug clinical trials in asthma, so the ability to refine these findings into actionable hypotheses are modest. One early study was that of Li et al. [135] in which the sputum microbiome was examined in a cohort of mild and severe asthma patients divided, for phenotype purposes, based on a sputum eosinophil count >3% and sputum neutrophil count >61%. Select bacterial families, including *Actinomycetaceae* and *Enterobacteriaceae*, were more abundant in patients with the eosinophilic inflammatory phenotype. In a study of 23 patients with corticosteroid-free (and thus mild) asthma compared to 10 healthy controls by bronchoscopy, asthma patients with low endobronchial eosinophils had decreased alpha-diversity and increased beta-diversity compared to both those asthma patients with high eosinophils and the healthy controls [184]. Several genera were significantly depleted (e.g., *Aeribacillus*, *Halomonas*, and *Sphingomonas*) or enriched (e.g., *Actinomyces*, *Bacteroides*, and *Neisseria*) in eosinophil-low versus eosinophil-high patients. A recent related study from China examined “non-eosinophilic” versus eosinophilic asthma and demonstrated decreased alpha-diversity in the former [141]. The first AsthmaNet Microbiome study [137] found that asthma patients defined as T2-high on the basis of gene expression in epithelial cells collected on endobronchial brushings had a significantly lower bacterial burden compared to patients defined as T2-low on the absence of this expression (Fig. 5.1). In contrast, the BOBCAT trial failed to demonstrate an association between T2-high related genes expressed in epithelial cells and either microbial taxa or diversity [134]. On balance, patients with T2-high asthma may have select changes in their airway microbiome that might be useful in defining further the phenotype, though further explorations between the microbiome and gene signatures in larger studies would be welcome.

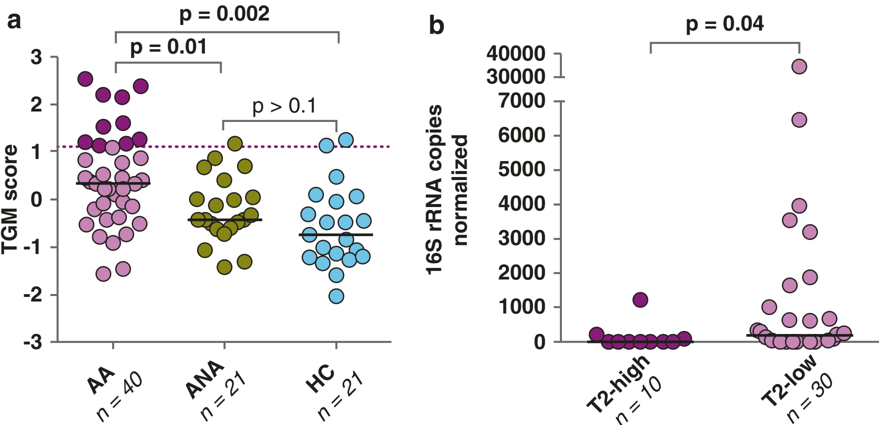

Fig. 5.1 The relationship of asthma, phenotype and atopy with bacterial load from the Asthma Microbiome I study. Figure (**a**) demonstrates the distribution of patients with asthma and atopy (AA), patients with asthma but no atopy (ANA), and healthy controls based on a gene scoring system using endobronchial brush samples. Using this, for the patients who are considered to be of the T2-high asthma phenotype (above the dashed line in (**a**) and to the left in (**b**), bacterial load is substantially lower than patients who are T2-low phenotype. With further validation, one could foresee that bacterial load could be used to help predict a correct phenotype for a patient with asthma: if high, the patient may have T2-low asthma. From reference (137) with permission

Patients with T2-low, neutrophilic asthma, defined in part as a ≥ 60% proportion of neutrophils in sputum, constitute a separate asthma phenotype. Defined generally by the absence of T2-high markers and by the presence of a higher proportion of sputum neutrophils, this phenotype incorporates about 60 to 75% of patients with asthma and is clearly heterogeneous [129, 185, 186]. This phenotype is said to be associated with more severe asthma and a poor response to corticosteroid therapy [187–189] (though the astute asthma clinician can readily find both milder cases in this phenotype and patients with T2-high asthma who are dependent on oral corticosteroids). Neutrophilic T2-low asthma may include patients with a “Th-17” phenotype, as higher concentrations of cytokines from Th-17 lymphocytes, such as IL-17A, IL-22, Il-23, TNF-α, and IL-8, can drive neutrophilic-predominant asthma [129, 179–183, 186, 188, 190–193], though a recent review questions the directionality of the association and suggests that IL-17 could be protective in asthma [194]. Examination of the airway microbiome in these patients generally demonstrates lower bacterial diversity and higher dissimilarity compared to those with eosinophilic asthma [146, 184, 187]. An early study from Wood et al. [195] examined airway neutrophilia in patients with asthma; patients with higher sputum neutrophil counts also had a higher load of potentially pathogenic bacteria as defined by culture. Likewise, in pre-school children with persistent wheezing who underwent bronchoscopy and BAL, those with peripheral airway neutrophilia (81% of all children), and a majority of these had elevated bacterial counts [196]. These studies suggested that neutrophilia and infection were linked in at least some cases of

asthma but of course could not suggest directionality. In more recent studies employing NGS, such associations have been demonstrated in more detail. One study examined patients with either severe asthma or moderate to severe COPD seen at the time of exacerbation and separated into clusters based on factor analysis of sputum mediators. In both diseases, patients with neutrophilic predominance by sputum and the presence of mediators such as Il-1β, IL-6, and TNF-α had increased proportions of Proteobacteria, whereas patients with eosinophilic predominance and the presence of IL-5, IL-13, and CCL26 had increased proportions of Bacteroidetes [197]. The study derived from the BOBCAT cohort demonstrated a positive correlation between Th17-associated genes in airway epithelium and several microbial taxa, particularly the increased abundance of Proteobacteria and that of families such as Pasteurellaceae, Enterobacteriaceae, and Bacillaceae [134]. Taylor et al. [187] showed that select genera, such as *Gemella*, *Rothia*, and *Streptococcus*, were decreased, as was alpha-diversity, in patients with neutrophilic versus eosinophilic asthma. In these patients, there was an inverse correlation between phylogenetic diversity and the proportion of sputum neutrophils. Yang et al. [146] demonstrated that patients with neutrophilic asthma had a higher bacterial burden that had less community richness and diversity, and had a taxonomic distribution that was distinct with an increased relative abundance of both *Haemophilus* and *Moraxella*, compared to patients with nonneutrophilic asthma. The patients with neutrophilic asthma also had higher concentrations of mediators such as IL-6, IL-8, IL-17A, and TNF-α that could drive increased airway neutrophilia.

These associations support the idea, yet to be proven conclusively, that patients with neutrophil-predominant, T2-low asthma have a dysbiotic microbiome that may drive the airway neutrophilia and contribute to the pathogenesis of this asthma phenotype. Infection and allergic inflammation may coexist, of course, and there are patients with both neutrophilic and eosinophilic asthma – a phenotype that is labeled as a "mixed" phenotype [128, 170, 181, 198]. The bacterial load, as defined using 16S rRNA gene copy numbers, is similar in the sputum of patients labeled with the mixed phenotype compared to the neutrophilic phenotype, and both are higher than that seen in patients who are "paucigranulocytic," with neither neutrophils nor eosinophils in sputum [146]. One interesting potential mechanistic explanation for this may be that allergic inflammation may promote bacterial persistence. Evidence for this comes from work by Essilfie et al. [199] in which mice were infected with live or killed *H. influenzae* and then sensitized and challenged with ovalbumin or placebo in a standard allergic inflammation model. This combination led to "persistent" airway inflammation, present 26 and 31 days after infection, while in an OVA-model alone inflammation typically has resolved. *H. influenzae* load was greater in the OVA-treated mice, and conversely, *H. influenzae* treatment suppressed some features of eosinophilic airways disease. More intriguingly, chronic *H. influenzae* infection combined with allergen challenge induced clear steroid resistance. A more recent study employing a similar mouse model with *H. influenzae* and OVA challenge demonstrated a similar induction of corticosteroid resistance which was accompanied by defects in regulatory T cell (Treg) associated immunosuppression,

airway remodeling, and goblet cell hyperplasia [200]. Taken together, these data clearly suggested that the combination of infection and allergen challenge promoted more chronic infection, changed the nature of the allergic airway inflammation, and elicited glucocorticoid resistance. The "mixed" phenotype that combines features of T2-high and T2-low (or perhaps better said, T2-high and T17-driven) then could be influenced in part by the lung microbiome.

One recent paper of potential interest to the asthma microbiome community examined the ability to stratify risk for COPD exacerbations by assessing the ratio of the relative abundance of Gammaproteobacteria and Firmicutes in serial sputum samples collected at the time of exacerbation. This "G/F ratio" revealed three separate groups of patients by cluster analysis; one group designated "HG" with a predominance of Gammaproteobacteria had a G/F ratio that correlated positively with increases in select inflammatory markers such as C-reactive protein and IL-1β over baseline, and negatively with FEV1 [201]. This study shows the potential of using the microbiome as a diagnostic tool to identify patients who then might be treated appropriately (or at least, differently). Such studies are very much needed in asthma.

Taken together, measures of airway bacterial burden, diversity or specific compositional features may help sharpen the distinction between T2-high and T2-low asthma phenotypes. However, the specific use of microbial markers in this regard, and their combination with inflammatory markers such as blood eosinophils, FeNO, and sputum cell counts or mediators, has not yet been formally done. Further, a dysbiotic microbiome may contribute to inflammatory changes and perhaps clinical outcomes, particularly with regard to the T2-low phenotype variant that is neutrophilic asthma. Larger, longitudinal studies that incorporate appropriate markers and microbiome analysis will be needed, as will more mechanistic studies in appropriate animal models. In particular, the question of whether dysbiosis drives neutrophilic asthma or whether neutrophilic asthma creates an ecological niche in which select microbiota can thrive, needs to be addressed.

The Obesity-Asthma Phenotype and the Microbiome One T2-low phenotype that at least somewhat separates from other phenotypes, particularly for severe and exacerbation-prone asthma, is that associated with obesity. Several epidemiological studies have suggested that obesity predisposes to asthma [202–205]. The effects of obesity on asthma prevalence [206–208], severity [209, 210] and response to treatment [208], including bariatric surgery [211], are complex. Obesity influences several asthma phenotypes [169, 208, 212] and response to asthma controller therapies [213–215]. Whether obesity causes a distinct asthma phenotype or whether it simply worsens pre-existing disease, either by changes in lung mechanics or by a change in airway inflammation, has been controversial, but growing evidence suggests that inflammation and oxidative stress may link obesity with asthma [215–221]. The adipocyte secreted hormones adiponectin (in its high molecular weight form, HMW-APN) and leptin are key regulators in long-term body weight, energy homeostasis, and fatty acid oxidation. Adiponectin-deficient mice have increased inflammatory cell infiltration in airways after allergen challenge [222]; a similar effect is seen after ozone exposure that is reversed with adiponectin expression

[223, 224]. Leptin is pro-inflammatory, and increases in leptin levels are associated with airway hyperreactivity and pro-allergic responses [225, 226]. Higher leptin and lower APN levels are seen in adult asthmatics [227]. Taken together, there is a clear association of adipokines, particularly leptin and APN, with asthma. Obesity associated with asthma declares later in life in adults, particularly in women [205, 208, 228], associates with asthma symptoms and exacerbations, with low expression of T2-high associated biomarkers such as blood eosinophils, serum IgE, and FeNO [210, 211, 229–231], and with systemic inflammatory markers such as IL-6 [232, 233] and C-reactive protein [233]. These latter markers and the absence of the T2-high markers suggest a T2-low phenotype.

Few studies have specifically examined the lung microbiome in obesity in general, or specifically obesity and asthma. The previously noted BOBCAT study showed a significant association of obesity (BMI > 30) with a distinct lung microbiome with a higher relative abundance of Bacteroidetes, Firmicutes, *Prevotella*, and certain *Clostridium* species, and lower abundance of Proteobacteria [134]. Endobronchial specimens of the obese subjects showed fewer mucosal and submucosal eosinophils compared to the lean subjects that paralleled the changes in the microbiome. Regrettably, larger studies have not yet specifically addressed the airway microbiome in obesity. One recent study examined the fungal microbiome (next section) and included a follow-up analysis of the bacterial microbiome described by Denner et al. [18]. While this analysis did not identify any significant association between BMI and any bacterial taxa, there were positive relationships between predicted functional bacterial pathways, such as galactose metabolism and linoleic acid metabolism, and BMI [234].

The data to date then suggest a potential role for the airway microbiome in obesity-associated asthma. Larger studies that delineate more precisely the relationship between the microbiome and adult patients with the obesity-associated T2-low asthma phenotype would be welcome. Especially important would be longitudinal studies that relate successful weight loss, either medical or surgical, with a change in the lung microbiome and concomitant improvement in asthma control.

Fungal Microbiome in Asthma

The microbiome of any ecological space in the human body will include more than just bacteria. Just as we expect to inhale and aspirate bacteria on a near-continuous basis, we may expect constant exposure of the upper and lower airways to fungi and the development of a fungal *mycobiome* within the larger microbiome. Fungi are eukaryotes, and the genera most commonly associated with allergy in humans are *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*. Fungi produce spores, or conidia, that can remain dormant until they are ready to germinate. Mesophilic fungi such as *Alternaria* and *Cladosporium* grow best at temperatures between 20 and 30 °C and thus ordinarily do not germinate in the body; they instead cause

respiratory allergies. Thermotolerant fungi such as *Aspergillus*, *Candida*, and *Penicillium* grow well at 37 °C and thus may elicit both allergy and grow well in the lungs, causing infection. This makes fungi unique compared to other allergens (e.g., pollens) and eukaryotes (e.g., house dust mites) that do not grow within the host. Inhalation of fungal spores, their fragments or their secreted products then may elicit lung disease. Larger spores such as *Alternaria* generally deposit in the upper airways whereas smaller spores such as *Aspergillus* may reach the small airways. In either case, germination, survival and growth then contribute to the mycobiome.

Fungi may also be found in the GI microbiome and are delivered by oral secretions or by food, and are generally considered to be transient and not colonizing [235]. Dysbiosis of GI fungi may have a role in early-life development of asthma and may be co-associated with bacterial dysbiosis [109, 112]. Mice treated with oral antibiotics that remove bacteria, particularly *Lactobacillus*, may have a fungal overgrowth that then can elicit an exaggerated response following airway antigen challenge via M2-macrophage polarization [236]. Fungus-free mice in which commensal or dysbiotic fungi are introduced into the GI tract can develop Th2-mediated for the former, and Th17-mediated for the latter immune responses in allergic airway inflammation [237]. In this way, a dysbiotic GI fungal community can influence distant lung immune responses.

Fungi are ubiquitous outdoors [238]; of those that may be important to asthma pathogenesis, *Cladosporium* and *Alternaria* are prominent [239]. Fungi can be found indoors in homes and offices in appreciable numbers. Older homes and homes with a "damp" indoor environment have a higher fungal burden, particularly for *Aspergillus* and *Penicillium* [240–242]. Each of these can be associated with asthma. Dust collected from homes can be rich in fungi and has a distinct fungal microbiome; this richness correlated with the age of the home and the relative humidity as well as with dog ownership [60], may be influenced but by outdoor air, and in contrast to the bacterial microbiome, less influenced by human occupants [60, 243, 244].

As with bacteria, at one point the lung was not considered to harbor fungi under normal conditions, as fungi could not be cultured [245]. It has long been appreciated that fungal sensitization is seen in at least some patients with asthma [246] and that there is a clear association between fungal allergen sensitivity and the presence of asthma and other respiratory diseases [247], particularly with *Alternaria* and *Aspergillus* [248–251]. At one extreme end this is manifested as allergic bronchopulmonary aspergillosis (ABPA), which may be considered as an allergic response to the fungus in the airway. ABPA presents as severe or poorly controlled asthma with a high serum IgE concentration, persistent eosinophilia, and bronchiectasis [252, 253]. These patients will have an elevated *Aspergillus*-specific IgE, detectable *Aspergillus*-specific IgG, and a positive skin-prick test for *Aspergillus* [254, 255]. There are patients with severe asthma who will meet only some of these criteria, or who are sensitized to a fungus other than *Aspergillus*; such patients may be sensitized (demonstrated by skin-prick testing or by blood allergen tests) to several genera of "environmental" fungi such as *Cladosporium*, *Alternaria*, *Penicillium*, *Candida*, and *Trichophyton* that are common in air and soil [256]. The terms "fungal asthma" or "severe asthma with fungal sensitization" (SAFS) have been used in

these cases that excludes ABPA [257]. While much attention is correctly paid to indoor mold and fungus exposure [241], particularly during infancy and early-life development [240, 258, 259], airborne outdoor fungi also can trigger asthma exacerbations in children and adolescents, particularly those already sensitized to *Cladosporium* [74].

Fungal products can promote allergic responses by virtue of being allergens and pathogen-associated molecular pattern (PAMP) molecules. These responses link a dysbiotic mycobiome to the inflammatory changes in asthma. Recent reviews of these products and their mechanisms have been published [260–263].

A dysbiotic lung mycobiome may be present in human asthma. As sequencing of the ITS1 (internal transcribed spacer 1) region of 18S-rRNA has become available and as reference libraries to interpret this sequencing, and to exclude human and other eukaryotic 18S, have become more complete, one can investigate the presence of the mycobiome in the lung. Van Woerden et al. examined the presence of sequenced fungi in sputum from patients with asthma and normal subjects; the former had a doubled-incidence of mold in the home. *Grifola sordulenta, Malassezia pachydermatis, Psathyrella candolleana, Termitomyces clypeatus* had a higher relative abundance in the sputum of asthma patients, while *Cladosporium cladosporioides, Eremothecium sinecaudum, Systenostrema alba,* and *Vanderwaltozyma polyspora* had a higher relative abundance in the sputum of control subjects. *Malassezia pachydermatis* is associated with atopic dermatitis [264], suggesting a role in atopy. Bronchoalveolar lavage done in 15 children with severe asthma with and without known fungal sensitization showed an increased abundance of fungal genera such as *Rhodosporidium*, *Pneumocystis*, *Leucosporidium*, and *Rhodotorula* compared to that seen in 11 normal children [265]. Interestingly, they did not detect an increased prevalence of environmental fungi such as *Aspergillus* and *Alternaria*. Bronchoalveolar lavage done in young to middle-aged adults with ABPA, SAFS, asthma without evidence of fungal sensitization, and control subjects noted that the healthy subjects had a low fungal burden with an abundance of *Malasezziales* [266]. In contrast, asthmatic patients with or without fungal sensitization or ABPA had an increased burden of *A. fumigatus* complex. The load of this fungus differed little between patients with a current history of itraconazole therapy versus no therapy; whereas patients with past history had a higher load. Both total fungal burden and the load of *A. fumigatus* complex were higher in those asthma patients receiving oral or inhaled corticosteroids. This study made clear the potential role and burden of *Aspergillus* in patients with severe asthma, even those without known fungal sensitization or ABPA.

A new study has now examined the potential interactions of the fungal and bacterial microbiomes in asthma with an emphasis on the T2-high phenotype. Sharma et al. [234] analyzed ITS1 sequences in endobronchial brushes and BAL samples from 39 asthmatic subjects separated by T2 and atopy status and 19 control subjects previously reported for their bacterial microbiome [18]. Asthma subjects with markers for T2-high disease had a lower fungal alpha-diversity than T2-low subjects and control subjects, and beta-diversity also differed based on T2 status in endobronchial samples. As with the bacterial microbiome, there is a fungal mycobiome in

normal, healthy subjects that is small in biomass. In BAL fluid, the relative abundance of *Trichoderma*, *Alternaria*, *Cladosporium*, and *Fusarium* species were significantly enriched in asthmatic patients, while *Blumeria* species, *Mycosphaerella* species, and different *Fusarium* species were enriched in healthy control subjects. Differences in endobronchial brushes were more modest, with an increased relative abundance of *Penicillium* in asthmatic subjects and those with atopy, and *Trichoderma* was increased in T2-high asthmatic patients. Seven key fungal genera – *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Penicillium*, *Trichoderma*, and *Mycosphaerella* – were significantly associated with asthma, T2 inflammation, and atopy. The authors then examined co-occurrence networks to examine differential associations between fungal and bacterial taxa. Samples from endobronchial brushes had a greater density of connections that maintained highly connected sets of taxa compared to BAL, and in each sample set, asthmatic patients had greater connections compared to controls. Examples of these are given in Fig. 5.2. From these networks, select fungal genera were associated with select bacterial genera. Although the keystone fungal taxa remained similar between BAL fluid and EB samples, co-occurring bacterial taxa were distinct between the two regions in asthmatic patients. Further, using a random forest model, the authors could identify top discriminatory fungal taxa, particularly *Alternaria*, *Cladosporium*, *Mycosphaerella*, and *Aspergillus*, that could classify asthmatic and healthy subjects with up to 72% accuracy. This study makes clear that fungal load, in addition to bacterial load here and in the study by Durack et al. [137], and the presence of select fungal and bacterial taxa can help to differentiate T2-high from T2-low asthma. These studies need repetition in larger and more varied patients, but the promise of being able to apply the lung microbiome as part of the delineation of asthma phenotypes and endotypes is becoming increasingly clear.

Specific Anti-Fungal Therapies in Asthma In patients with asthma with either ABPA or fungal sensitization, antifungal therapy with itraconazole or similar azoles can be a useful adjunct [267–269], and this forms part of the evidence base for the potential role of the mycobiome in asthma, even as *Aspergillus*, as previously noted, may not be present or even in substantial prevalence in some patients with fungal asthma. Several trials have looked at the utility of these therapies in severe asthma in the absence of clear evidence of ABPA. Denning et al. [257] conducted a randomized, placebo-controlled trial of itraconazole, an agent with a wide spectrum of anti-fungal activity, in 58 adult patients with severe asthma and SAFS. These patients had been receiving or had recently received oral corticosteroids, were skin-prick test positive for one of several fungi, were negative for *Aspergillus* precipitins (IgG), and had a circulating IgE concentration of less than 1000 IU/ml. Other asthma therapies were optimized before enrollment. Patients receiving anti-fungal therapy had a significant improvement in asthma quality of life scores and a decrease in total IgE concentrations. Interestingly, relapse in symptoms after discontinuation of itraconazole was common. Other azole agents also have noted improvement in quality of life scores for patients with SAFS [270, 271]. A more recent study examined another potent anti-fungal agent, amphotericin B, as nebulized therapy in 21

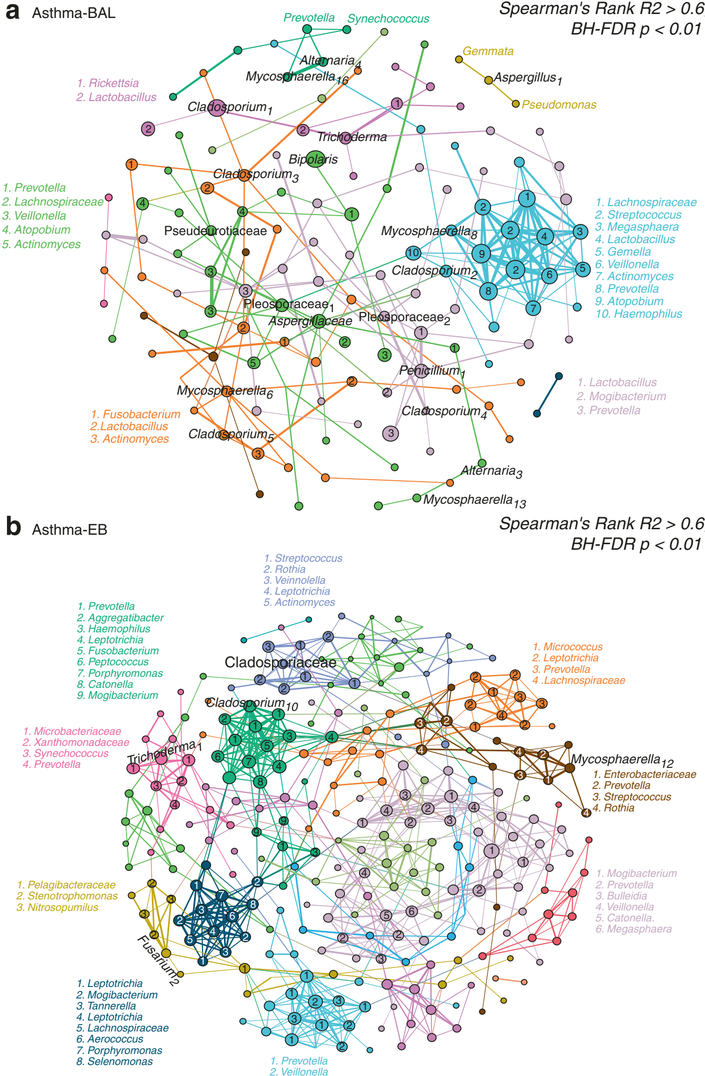

Fig. 5.2 Significant co-occurrence relationships of fungi and bacteria between different modules in patients with asthma using samples collected from BAL fluid (**a**) or endobronchial brushes (**b**). These networks represent statistically significant correlations; a connection stands for a strong (Spearman $\rho < 0.6$) and significant ($P < 0.01$) correlation. Fungal nodes are labeled in black inside the network, and bacterial nodes are labeled outside the network in different colors depending on the module to which they belong. Nodes are colored by modules or communities (group of taxa) based on Louvain community detection algorithm. The size of the node is controlled by the number of connections). The edge width is proportional to the weight of correlation. The level of modularity ranges from 8 to 16 for all networks. For genera with more than 1 differentially abundant taxa, the ESV number is shown as a subscript. Co-occurrence relationships could be used to determine which bacteria and fungi might be predicted to be present in the airway microbiome when one or more are detected in a clinical sample, and could be used (for example) as an aid to determining a correct phenotype for a patient with asthma. From reference (234) with permission

patients with severe asthma who were labeled either as SAFS or had a diagnosis of ABPA. In contrast to the Denning study, the response rate as measured by quality of life improvement was less than 15% and substantial side-effects, including bronchospasm were noted [272].

Effects of Asthma Medications on the Airway Microbiome

As we begin to consider longitudinal studies of the airway microbiome in asthma in both children and adults, two immediate questions arise: first, how might asthma-related medications change the microbiome for better or for worse, and second, might we introduce medications that would change the microbiome and thereby improve asthma control?

Corticosteroid Therapy and the Airway Microbiome Inhaled corticosteroids are a mainstay of asthma controller therapy, and oral corticosteroids are near-universally employed in the treatment of severe acute exacerbations. While these agents have no antimicrobial activity per se, inhaled steroid therapy often ameliorates underlying airway inflammation, and this may alter the local microbiome. Early studies of the microbiome in asthma were small and generally included patients receiving inhaled corticosteroids, and therefore it was not possible to separate a steroid-specific effect. Goleva et al. noted no differences between corticosteroid-resistant and corticosteroid-sensitive patients with asthma at the phyla level regarding diversity, richness and taxonomy, but select genera, including *Haemophilus*, were increased in the steroid-resistant group [131]. Denner et al. demonstrated increased Proteobacteria and decreased Bacteroidetes, and increased *Pseudomonas* and decreased *Prevotella* and *Veillonella*, in patients received inhaled corticosteroids versus those without such therapy [18]. From these studies it becomes clear that the airway microbiome is perhaps different in patients taking inhaled corticosteroids as a controller therapy for asthma. The question then becomes, does the addition of such therapy change diversity or taxonomy? Or is the necessity for use of corticosteroids a marker for more severe underlying disease that is itself responsible for the changes, or do the corticosteroids elicit a host response that then alters the microbiome? In the AsthmaNet Microbiome Study [137], a subset of recruited patients with steroid-naïve asthma received inhaled fluticasone or placebo for 6 weeks, with bronchoscopy and sputum collection before and after therapy. Patients who responded to steroid therapy with an improvement in methacholine responsiveness (ICS responders) were compared to nonresponders; the former had a baseline bacterial microbiome more similar to that of healthy controls with an enrichment of Streptococcaceae, Fusobacteriaceae, and Sphingomonodaceae, whereas nonresponders at baseline were enriched in Microbacteriaceae, Pasteurellaceae, and asthma-associated *Haemophilus* genera. Microbiome analysis of endobronchial brush samples was limited by insufficient 16S rRNA amplicon in paired samples (pre- and post-intervention) from some subjects. At the taxon level ICS treatment resulted in

increased relative abundance of Microbacteriaceae and *Neisseria* and *Moraxella* species, and depletion of a specific *Fusobacterium*, which was not observed with the placebo treatment [137]. Parallel observations including differential changes between the two treatment groups, before and after the interventions, were observed in a subsequent analysis of induced sputum from participants in the same study [136]. Two points can be made here based on the studies conducted to date. First, inhaled corticosteroid therapy in patients with mild asthma and who respond to such therapy clearly have some changes in airway taxonomy. Second, much larger trials are needed if we are to determine better the changes in the microbiome after initiation of corticosteroid therapy, and the interplay of such a treatment effect compared to changes in host inflammation elicited by corticosteroid therapy.

Corticosteroids are potent inhibitors of pro-inflammatory molecules and cells, and it is tempting to suggest that glucocorticoids would also alter expression and production of innate immune responses that generally prevent infection. This in fact does not occur and indeed, corticosteroids generally fail to inhibit the expression of many of the genes involved in innate immunity [273]. In airways, the epithelium produces a number of innate immunity-related proteins that destroy or suppress microorganisms, including complement, collectins, lysozyme, lactoferrin, secretory leukocyte protease inhibitor, and defensins. Defensins, small cationic proteins that are expressed either constitutively or can be induced by various pathogens [274], are regulated by Toll-like receptors (TLR) such as TLR2 [275, 276]. Corticosteroids may enhance Toll-like receptor (TLR) 2 expression, the absence of which leads to an inability to clear organisms such as *Mycoplasma* and an inability to induce human β-defensin [277, 278]. Another anti-microbial protein is CCL20, also known as MIP-3a, that is similar to defensins. CCL20 is expressed in the airway epithelium and can be induced by bacteria, and is regulated by several TLRs [274, 279, 280]. In cultured airway epithelial cells, treatment with corticosteroids enhances the production of CCL20 [281, 282]. Treatment of airway epithelial cells with budesonide but not fluticasone elicits increased expression of both CCL20 and lactotransferrin [283]. One older study demonstrates that corticosteroids increase secretory leukocyte protease inhibitor transcripts in airway epithelial cells [284].

Counterbalancing the idea that corticosteroid therapy may be benign or even beneficial with reference to the lung microbiome in asthma are data from a number of clinical trials in COPD that inhaled corticosteroid treatment can increase the risk of pneumonia [285–290]. In the few studies to date that report asthma patients separately from COPD with regard to pneumonia incidence, little increased pneumonia risk is seen with the use of inhaled corticosteroids in asthma: for example, the START trial (*Steroid Treatment As Regular Therapy*), with over 7200 patients demonstrated no increased risk of pneumonia in patients treated with budesonide compared to placebo [291]. Two smaller randomized trials of either fluticasone [292] or budesonide demonstrated similar findings [293]. A recent meta-analysis of the risk for pneumonia in patients with asthma has reported little if any increased risk [294]. The mechanisms by which corticosteroids then alter the airway microbiome are not yet clear, but data to date suggest that by preserving and perhaps even enhancing

select innate immunity responses that are critical to fighting bacterial colonization, corticosteroids may change the microbiome in some way that is beneficial in asthma.

Beta-Adrenergic Agonist Therapy and the Airway Microbiome Inhaled beta-adrenergic agonists, short-acting or long-acting, are a mainstay of therapy in asthma. To date, there are no reports of the effect of these drugs specifically on the lung microbiome. In any study or experiments in which these agents are co-administered with inhaled corticosteroids, one cannot exclude a modulating effect., perhaps by interactions on genes targeted by the glucocorticoid receptor [295, 296].

Macrolide Antibiotic Therapy and the Airway Microbiome Antibiotics are commonly given to patients with asthma exacerbations, even as there is little evidence of their efficacy [297]. Recognition that at least some patients were colonized with bacteria such as *Chlamydophila pneumoniae* or *Mycoplasma pneumoniae* that contributed to their asthma symptoms [298–303] led to the idea that perhaps antibiotic therapy directed against these organisms would be useful therapy. The most studied antibiotic in asthma to date has been the macrolides.

Macrolide antibiotics, from the parent erythromycin to currently available agents including azithromycin and clarithromycin, are one of the most widely used antibiotic classes and have a role in the treatment of other obstructive airways diseases such as COPD, cystic fibrosis, non-CF bronchiectasis, and bronchiolitis [304–310]. As noted above, chronic asthma may be triggered repeatedly by viral respiratory infection and by select bacteria such as *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae*, and this led to a suggestion that macrolide therapy might be useful in asthma. Further, macrolides have potential immunomodulatory and antiviral properties [311, 312], perhaps via suppression of NFκB [313–316], that extend beyond their role as direct antibacterial agents. Examples that are relevant to the airways and asthma include the ability of azithromycin therapy to maintain airway epithelial barrier integrity in culture models of *Pseudomonas* infection [317], which as noted earlier in this chapter may be found in increased relative abundance in asthmatic airway microbiome, suppress biofilm formation by *Pseudomonas aeruginosa* [318], and blocks quorum sensing by *P. aeruginosa* [319]. Macrolides inhibit mucin formation in cultured, differentiated airway epithelial cells induced by *Fusobacterium nucleatum* that is independent of any anti-bacterial activity [320]. Azithromycin can inhibit epithelial cell apoptosis and the epithelial-to-mesenchymal transition that occurs with ovalbumin challenge in mouse models [321, 322]. Despite the risks of anti-macrolide resistance that could develop from long-term use [323], these potential nonbactericidal mechanisms combined with the potential for direct action have led to exploration of macrolide therapy as a potential adjunct asthma therapy.

Given the scientific rationale for their use, their efficacy in clinical asthma trials has been variable and until recently disappointing. From the 1950s to 1970s, studies suggested that the macrolide troleandomycin could be a steroid-sparing agent in patients with severe asthma receiving parental corticosteroids, perhaps by blocking metabolism of methylprednisolone [324–327]. One trial demonstrated that azithromycin was unlikely to be a steroid-sparing agent in children with moderate to severe

asthma who were receiving high dose inhaled corticosteroids; however, this trial had difficulty recruiting participants and was prematurely terminated for futility [328].

As "atypical" bacteria were recognized to be present in at least some children or adults with asthma, consideration was given to the antibiotic effect of macrolides. Here, results were inconsistent: for example, Kraft et al. [301] demonstrated that clarithromycin treatment increased FEV1 in patients who had evidence of either *M. pneumoniae* or *C. pneumoniae* infection based on PCR analysis of upper and lower airway samples. Against this, a report from the Asthma Clinical Research Network demonstrated that clarithromycin therapy did not improve asthma outcomes in those patients with suboptimally controlled asthma [329]. This trial was stratified based on PCR evidence for the same two microorganisms, and neither group improved. These trials required evidence of bacterial infection by either culture or PCR demonstration, and both were insensitive compared to next-generation sequencing. Similar trials examined different macrolide antibiotics generally over six to 26 weeks with assessment by clinical markers, and these trials in adults had varied results [330–337]. As recently as 2015, a Cochrane systematic review was inconclusive as to the clinical use of macrolides in asthma [338].

More recent studies took advantage of our improving understanding of asthma phenotypes. The recognition of neutrophilic asthma as an asthma phenotype, the suggestion that this phenotype was associated with increased bacterial load and potential pathogens such as *Haemophilus influenzae* and *Moraxella catarrhalis* and with neutrophil-associated cytokines such as IL-8 [195], and the understanding that neutrophilic inflammation was crucial to the pathogenesis of panbronchiolitis in which macrolide antibiotics had a demonstrated role [310, 339], all suggested a role for macrolides in neutrophilic asthma. Simpson et al. [333] demonstrated that clarithromycin therapy decreases both the number of neutrophils and concentrations of IL-8 in the sputum of patients with severe asthma. A randomized clinical trial (*AZISAST*) compared the effectiveness of azithromycin for prevention of exacerbations in severe asthma among patients treated daily for 6 months. The primary endpoints, that of the rate of severe exacerbations and lower respiratory tract infection, were not met overall, but in pre-defined patients with non-eosinophilic asthma (blood eosinophils <200/μl and fractional excretion of nitric oxide below the lower limit of normal), patients with at least one primary endpoint event decreased from 62% in the placebo group to 33% in the treated group, a relative risk reduction of 54% [331]. To the extent that neutrophilic, non-eosinophilic asthma might be driven by the microbiome, these data suggested that macrolide therapy could be useful in treatment.

As next-generation sequencing became available, the question of whether macrolides could change the airway microbiome directly and thus change asthma control could be asked. One very early small trial examined the lower airway microbiome sampled by bronchial washing for and six weeks after treatment with daily azithromycin; therapy was associated with decreased richness and reductions in *Pseudomonas*, *Hemophilus*, and *Staphylococcus* [340]. In a follow-on to the AZISAST study [331], Santiago et al. [341] examined the oropharyngeal microbial

community in 13 patients with moderate to severe asthma (8 receiving azithromycin, 5 receiving placebo) at baseline, during and after 6 months treatment with either azithromycin or placebo. They found that the overall composition of the oral microbiome in these patients differed little to that of the healthy population. Treatment over 6 months with azithromycin increased the relative abundance of S*treptococcus salivarius* and decreased that of *Leptotrichia wadei*. The authors noted that they used oropharyngeal samples out of concern that collection of lower airway samples might induce asthma, a concern not borne out in other studies. Another recent study that demonstrated the utility of azithromycin as adjunct therapy for both T2-high and T2-low severe, uncontrolled asthma was the AMAZES trial, a placebo-controlled, randomized clinical trial in which 48 week therapy with thrice-weekly azithromycin reduced asthma exacerbations and improved quality of life [342]. Subsequent analysis of sputum samples from a subset of these patients demonstrated that azithromycin treatment did not alter bacterial load, nor alter the relative abundance of select pathogens such as *Moraxella* and *Pseudomonas*, but did decrease Faith's phylogenetic diversity index and decreased the relative abundance of *Haemophilus* [343]. One concern in this study was a noted increase in several macrolide resistance genes as noted by PCR analysis of sputum. Taken together, the microbiome analysis performed from the AZISAST and AMAZES studies begins to make clear that whatever the effects of azithromycin are on asthma, its effects on the microbiome appear modest. This raises the question as to whether the immunomodulatory effects of this antibiotic are more important.

Other clinical trials outside of asthma have examined the effect of macrolides on the airway microbiome. Azithromycin therapy during the treatment of respiratory syncytial virus bronchiolitis infection in infants decreased the abundance of *Moraxella* in nasal lavage samples collected pre and post treatment [344]. Other bacterial taxa were unchanged by treatment, and the lower abundance in *Moraxella* was associated with less respiratory wheezing in the ensuing twelve months. Likewise, treatment with erythromycin for 48 weeks in 84 adults enrolled in the *Bronchiectasis and Low-dose Erythromycin Study* (BLESS) demonstrated lower abundance of *Actinomyces* and *Streptococcus* but an increased abundance of *Haemophilus* in oropharyngeal swabs [345]. In COPD, azithromycin therapy has been considered as among first-line therapies for treatment of exacerbations [346, 347], and as a long-term adjunct treatment of GOLD class 4 COPD [348–350]. One study examined the lower airway microbiome by BAL in 20 patients with COPD before and eight weeks after treatment with either daily azithromycin or placebo. In this trial, azithromycin treatment did not alter bacterial burden but did decrease the relative abundance of a number of taxa, alpha-diversity, and concentrations of chemokines and cytokines such as CXCL1, TNF-α, and IL-13 [351]. As in the asthma studies, the effects of macrolide therapy on the microbiome were modest.

In summary, it becomes clear that macrolide therapy has complex effects on the lung, including on the lung microbiome. These changes may be due both to the direct, antimicrobial effect on the bacteria and by more indirect effects as an immunomodulator, with the latter perhaps being the more important. Resolving these questions may provide a better rationale for the use of macrolide therapy in asthma.

Other Antibiotics and the Airway Microbiome Lastly, older studies have examined the use of penicillin-based antibiotics in patients hospitalized for asthma exacerbations and found no evidence for efficacy [352, 353]. To date, there are no NGS-based studies that examine the role of penicillin-based antibiotics, or of other antibiotics commonly employed in outpatients with respiratory symptoms (e.g., sulfonamides, cephalosporins, tetracycline) on the airway microbiome in asthmatic patients.

Probiotics and the Airway Microbiome The GI microbiome has become an important and increasingly studied etiological factor in a number of immunological, neurological, and malignant diseases. As noted earlier, there is increasing recognition of how the gut-lung axis could influence T cell plasticity and function and dendritic cell function. While much attention has been focused on the gut-lung axis in early life, one recent study has demonstrated differences in the gut bacterial community structure in a group of adults with asthma compared to control subjects without known lung disease [168]. Differences in specific gut bacterial communities at the phylum level (Bacteroidetes and Firmicutes) associated with FEV1, and select OTUs were either decreased (Bacteroides, Enterobacteriaceae) or increased (*Bifidobacterium*, and Lachnospiraceae) in subjects with asthma. Cluster analysis done by variation in gut bacterial community structure demonstrated three different clusters in asthma patients; one such cluster had greater airway hyperresponsiveness and bronchodilator reversibility. Thus, the GI microbiome could be used as part of a phenotyping assessment of asthma patients.

For these reasons, modulating the adult GI microbiome by probiotic therapy with live microorganisms that alter these functions in favor of asthma control and better health, is an attractive consideration, and one that might avoid many of the problems associated with antibiotic use.

Lactobacillus is one probiotic that has received considerable attention. A low relative abundance of *Lactobacillus* has been shown to be associated with early development of allergy in infants [354–356]. Oral supplementation with different *Lactobacillus* species, particularly *L. reuteri*, alleviated airway inflammation, decreased IgE production, and decreased production of T2-associated cytokines induced by house dust mite allergen in a mouse model [357]. Likewise, oral gavage with *L. paracasei* L9 attenuated airway hyperresponsiveness, eosinophil infiltration in airways, and decreased serum IgE after exposure to particulate matter 2.5 (PM2.5) in mice sensitized and challenged with ovalbumin [358]. Treatment of ovalbumin sensitized and challenged mice with *Lactobacillus rhamnosus GG* (LGG) by gavage improved both airway inflammation and airway remodeling as evidenced by reduced collagen deposition and expression of markers such as T-bet, GATA3, and Foxp3 [359]. These animal studies clearly establish a potential use for probiotic therapy in airway inflammation.

Trials in which infants at high risk for atopic disease (usually by virtue of having one or two atopic parents) have received probiotic therapy, usually *Lactobacillus rhamnosus GG* (LGG) by oral supplementation, have had varying results. One early

trial in Finland suggested a benefit by decreasing subsequent atopic disease, a benefit that extended to age 5 [360–362]. However, a different trial done at about the same time demonstrated no clinical benefit from LGG when given to pregnant women with a family history of atopic dermatitis in primary prevention of atopic dermatitis to their offspring [363]. A trial in which preschool children with allergic asthma or rhinitis consumed fermented milk containing *L. casei* demonstrated no improvement in asthma control [364]. Likewise, in the *Trial of Infant Probiotic Supplementation* (TIPS), oral administration of L*GG* supplementation for six months in 92 high-risk infants on the subsequent incidence of eczema (atopic dermatitis), with development of asthma examined as a secondary end-point of the study, failed to demonstrate a benefit for either disease [365]. In a follow-on study from the TIPS trial, Durack et al. [366] examined gut microbiota maturation in these infants, and demonstrated that LGG supplementation in infants with a "meconium" dysbiosis of their GI microbiome had increased diversification of their microbiota and increased production of anti-inflammatory lipids during LGG treatment. However, within six months of cessation of supplementation, all benefits had been lost. Thus it is not clear today whether probiotic intervention in early life conveys a lasting benefit in asthma and other allergic diseases.

In contrast, trials in children and adolescents with asthma suggest some benefit with probiotic therapy. An early trial of *L. gasseri* supplementation in children aged 6 to 12 years with asthma and allergic rhinitis over two months demonstrated improved pulmonary function compared to placebo [367]. A more recent placebo-controlled, randomized clinical trial examined supplementation of *L. paracasei*, *L. fermentum*, and their combination on asthma control over three months in 160 adolescents aged 6 to 18 years with asthma. Children continued usual care and asthma medications in this time. The children receiving *Lactobacillus* therapy in any combination had lower asthma severity and improved control compared to those children receiving placebo [368]. A small pilot trial from Brazil in 30 children aged 6 to 17 years with asthma received a probiotic containing *L. reuteri* or placebo; children receiving the probiotic over 60 days had an improvement in symptoms and asthma control test scores [369]. A recent meta-analysis of 17 randomized controlled trials in 5264 children suggested that LGG supplementation elicited a reduction in the occurrence of asthma but not other atopic diseases in post-natal periods [370].

One promising probiotic agent is OM-85 Bronchovaxom (OM-85 BV), a low-endotoxin alkaline extract of 21 strains of five pathogenic bacteria that on oral administration as a prophylactic agent reduces the frequency and duration of respiratory tract infection in children [371–374]. Used in pre-treatment, OM-85 BV prevents airway hyperreactivity and inflammation in *Leishmania major* antigen sensitized and challenged mice by increasing the number of regulatory T cells in the airway [375]. The recent EOLIA trial compared the effect of sublingual administration of a similar *polyvalent mechanical bacterial lysate* tablet versus placebo for three months in 152 children aged 6 to 16 years with asthma, a history of asthma exacerbations, and sensitivity to dust mite allergen. While the major endpoint of a change in ACT score was not met, the number of asthma exacerbations decreased in

children treated with the bacterial lysate [376]. Data regarding OM-85 BV in adults is sparse to date. The upcoming PrecISE precision intervention trial for severe asthma sponsored by NHBLI will examine the potential utility of OM-85 BV in two patient cohorts, adolescent and adult respectively, with severe asthma in an adaptive trial design.[1]

No other probiotic trials for adults with asthma are published to date.

Effects of Asthma Exacerbations on the Airway Microbiome

Asthma exacerbations can be triggered by a variety of environmental exposures; one major trigger is respiratory tract viral infection [377–379]. Children with asthma may have exacerbations triggered by bacterial pathogens such as *H. influenzae*, *M. pneumoniae*, *M. catarrhalis*, or *C. pneumoniae*, as noted previously, in addition to viruses. In contrast, respiratory bacterial infection is not commonly associated with asthma exacerbations in adults; this is a noticeable distinction between asthma and COPD [380]. While there are scant data to date on the lung microbiome in adults before and after exacerbation, the AZISAST and AMAZES trials with azithromycin suggest that modification of the lung microbiome may change the frequency of and susceptibility to exacerbations [331, 342, 343, 381], though the changes in the microbiome in the AMAZES trial were, as noted previously, modest and suggested that azithromycin may exert some of its protective effects via non-bacterial mechanisms.

There is some indirect evidence that viral infection may increase susceptibility to subsequent bacterial growth, and that this may be relevant to asthma. Viruses such as rhinovirus, influenza, and respiratory syncytial virus (RSV) can increase airway epithelial cell barrier permeability via disruption of tight junctions, alter expression of adhesion molecules that then may promote bacterial invasion, impair cilia function, decrease the expression or effectiveness of host defense proteins such as the Toll-like receptors and secretory IgA, and increase mucous production [382–388]. Indeed, it is clear that the epithelium is the principal site of first attack for most viral infections of the airway [389]. Any viral infection would also alter the local environment in terms of nutrient availability, oxidative status, and presence of mediators that may change bacterial virulence and survival [385]. Whether any of these events are important to asthma exacerbations in terms of susceptibility, risk or duration, or in a subsequent alteration of the airway milieu that in turn may promote a longer-term dysbiotic microbiome, is unknown.

[1] Information about the PrecISE trial can be found at https://preciseasthma.org/preciseweb/

Future Directions

We have learned much about the lung microbiome in health and in obstructive lung disease over the past decade. Next-generation sequencing, bioinformatics, mechanistic studies of the microbiome in mice, and the ability to gather increasingly large cohorts of human participants in clinical trials have increasingly demonstrated that the lung microbiome in asthma is dysbiotic and may well have a role in pathogenesis and disease progression in select asthma phenotypes. To date, much of this research, while increasingly elegant in research methods has been descriptive and based on single time-point observations.

There is a clear and compelling need to apply an ecological framework to understanding the pathogenesis of asthma and for longitudinal studies that are large and well organized with the following characteristics: collection of multiple microbiological specimens from the lung, upper respiratory tract and gut; additional biospecimens and genetic samples to work out biomarkers of disease and phenotype to deeply characterize host response factors, and collection of individual-specific environmental and behavioral data (Fig. 5.3). Such studies are needed involving multiple, diverse populations around the world, and at present are particularly lacking in the study of adult asthma. The single largest impediment to such studies is that no agency, government or private, has been willing to date to pay for the twenty-first century equivalent of a "Framingham" style asthma study. Such studies must surmount the substantial costs of collection and analysis and the significant burdens placed on the participants. Further, these studies must assume the task of accounting for differing environments, both indoor and outdoor, that may alter the microbiome (gut or lung) and the host defenses, seasonality, and the interactions of nonmicrobial allergens with the microbiome and with host responses. The human subjects of course have a varying genome and differences in the expression of each of the "-omic" areas that might be studied, as well as interactions between these -omics. In the end, these studies would need to provide specific etiologic, mechanistic, and associative insights about the microbiome and asthma so as to inform experimental cell-based and animal models of host-microbiome interactions, which then could come back to appropriate clinical trials.

If the microbiome has a role in asthma, it must do so by inducing a response in the human host. The large studies will need to correlate key features of the microbiome, and features that go beyond simple taxonomy or diversity, with host clinical, cellular, and molecular disease markers. As one example, endobronchial brushings collected from patients with and without malignant lung nodules, sequenced for both bacterial (16S rRNA) and host transcriptome profiles, demonstrated that patient transcriptomic signatures relevant to lung cancer pathogenesis were associated with increased relative abundance for *Streptococcus* and *Veillonella* in the brush samples [390]. Such a study done in patients with asthma of a carefully curated phenotype (e.g., the T2-low, Th-17 high phenotype that appears to be most correlated with a high bacterial burden, as previously discussed), combined with a

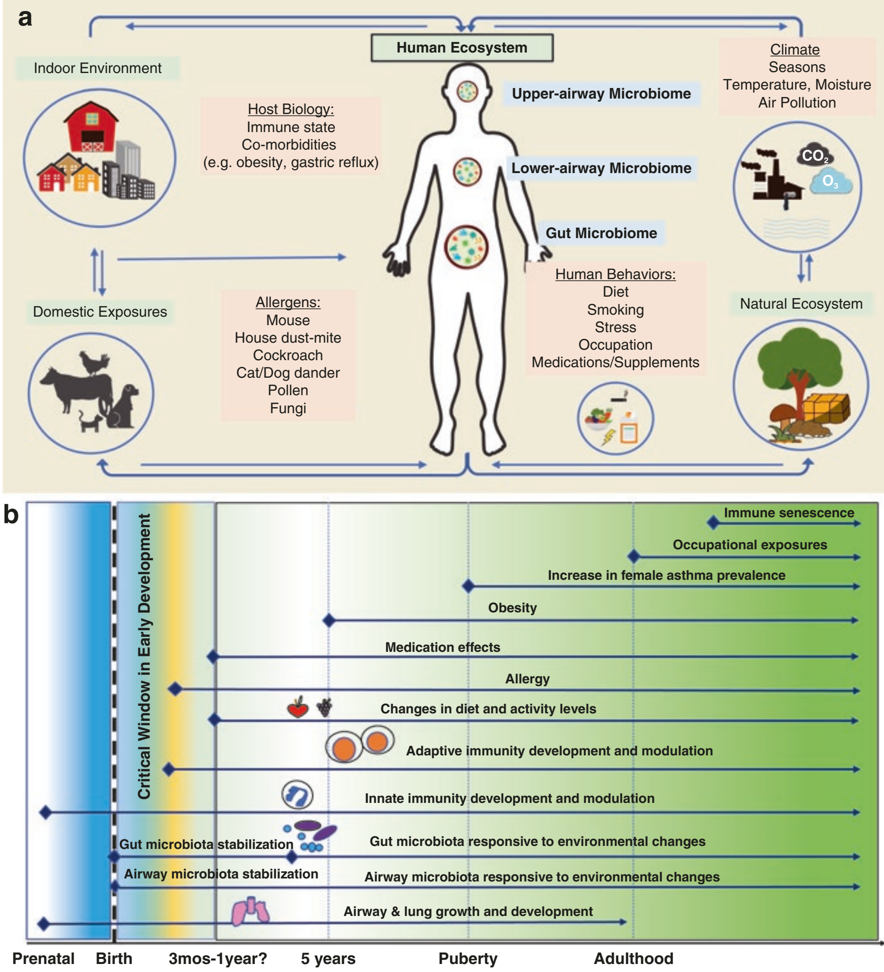

Fig. 5.3 (**a**) Ecological interactions shape both host biology and their microbiomes. The human body is a complex ecosystem that experiences concurrent microbial and nonmicrobial exposures from external/built environments, other animals, diet, medication, occupational exposures, and pollutants. The human ecosystem is also impacted by concomitant inflammatory and/or immune disorders An ecological framework is necessary to advance mechanistic insights into these multi-directional interactions and how they shape asthma. Adapted from reference (396) with permission. (**b**) The longitudinal nature of ecological interactions from prior to birth to adulthood that shape asthma risk, development, and clinical outcomes, with reference to the microbiomes and the relationships of microbiota to the immune system and key environmental influences. Adapted from reference (397) with permission

multiple -omics strategy could examine, for example, single nucleotide polymorphisms and micro-RNA on the host side and metagenomic analysis on the microbiome side, to test whether similar associations exist. Select differences then could be tested in newer dedicated cell- and organ-cultures, including cultures that use

engineered substrates populated with cells collected from airways or derived from stem cells, and "lung-on-a-chip" microsystems [391].

Animal models in which a human microbiome is transplanted into germ-free (GF) ("axenic") mice also provide a model in which causal relationships can be explored between the microbiome and a host. There is recognition that while the GF mouse, lacking its own microbiome, represents a "blank slate" [392–395] upon which human microbiota can be specifically tested, alone, in select combinations, or as a collected ecology from human subjects, to provoke host responses, particularly when combined with airway allergen challenge. There are clear differences between human and mouse immunology that must be taken into account in explaining the results of such experiments. Further, facilities to generate and care for these mice are frightfully expensive – these mice are literally worth their weight in gold. Such limitations for now limit these experiments.

Perhaps the greatest impediment to date has been one that is now finally disappearing – lung biologists now agree that the lung is not sterile. This recognition must translate to a recognition that large clinical trials should be sampling the lung and gut microbiomes as part of their design. Even if not analyzed at the time for the potential role in modulating the response to a pharmacological therapy, the use of these samples (as in the BOBCAT trial, [134]) in post-hoc explorations not only offer the potential to relate the microbiome to the studied phenotype or group of patients but also, by adding larger numbers of patients, provide important corroboration to our understanding.

Among the key questions to be addressed in the future is whether intervening in the adult microbiome in asthma matters. If the "critical window" in which the lung or gut microbiome (with other factors) influences the host immunology to develop an asthma phenotype is open only during early life, then modification of the microbiome in adults may be well beyond the time in which such modifications can have any useful effect. That the use of simple antibiotics such as azithromycin can modulate asthma clinical control suggests that this extreme and perhaps dour view of the microbiome is unwarranted, and that the microbiome is at least to some degree "plastic" and changeable. Modification of the microbiome need not be direct to be useful; changing the host immune system (e.g., even with inhaled corticosteroids) in ways that in turn modify the airway microbiome may elicit a useful downstream effect. Modification of the microbiome need not work in every adult with asthma to be useful: the asthma community is correctly vested in anti-IL-5 and anti-IL-4/IL-13 biologic therapy for the relatively modest number of patients with severe, T2-high asthma (15 to 30% of all asthma patients are T2-high, and 10 to 20% have severe asthma, so in the end we are addressing 2 to 6% of the asthma population). A microbiome-based therapy that addresses a phenotype presently unreachable, such as the "neutrophilic" phenotype that is T2-low and perhaps Th-17-high, would be a substantial advance.

The ultimate goal in understanding the lung microbiome in asthma is to uncover epidemiologic, diagnostic or therapeutic features that will improve patient care. The microbiome may aid us in delineating phenotypes and understanding markers of disease risk and progression. Not only might we find therapies that may modify the

microbiome (lung or gut or both) that improve clinical outcomes, as importantly, we might uncover beneficial or deleterious effects of current therapies. All these will shape our understanding of adult asthma and lead to more personalized care. Understanding the microbiome in asthma then is much like understanding the immunology, cell biology and epidemiology of this disease: a necessary part that will shape how we care for patients in the future.

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Chapter 6
Microbiome in Cystic Fibrosis

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Introduction

Cystic fibrosis (CF) is a life-shortening, autosomal recessive genetic disease that affects over 70,000 individuals worldwide [1]. Mutations in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene lead to dysfunction or absence of the CFTR protein, a cell membrane channel that regulates chloride and bicarbonate movement in epithelial cells lining the respiratory, digestive, and reproductive systems and sweat glands [2]. Phenotypically, CFTR dysfunction results in chronic airway infection and inflammation, nutritional and gastrointestinal (GI) disorders, male infertility, and increased salt loss from sweat, among other consequences [3]. Diverse airway pathogens, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Burkholderia* species, and other gram-negative bacteria, are well-known contributors to CF lung disease [4]. Chronic infection with these pathogens and excessive inflammatory response lead to progressive airway structural injury; most people with CF succumb to respiratory failure in early adulthood.

Microbiologic surveillance for respiratory pathogens is a routine part of CF clinical care [5]. Culture using standardized laboratory approaches is performed on

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respiratory samples such as oropharyngeal swabs (OP), spontaneously expectorated sputum, sputum induced with nebulization of hypertonic saline, and bronchoalveolar lavage fluid (BALF) [6, 7]. Sputum is the preferred specimen type for microbiologic surveillance (for reasons discussed below), but in individuals unable to expectorate, OP swabs are routinely used. Bronchoscopy with BALF collection is more invasive than other sampling modalities and is therefore generally reserved for those with suspected undetected pathogens. Sputum induction is used in research settings, and, in some centers, for clinical care but is not universally available [8]. Microbiologic culture of respiratory samples requires specific processing and culture media to maximize growth of suspected pathogens (e.g., *P. aeruginosa*) [5]. The focus on suspected pathogens creates a bias in culture detection, and unsuspected or fastidious microbes may remain undetected.

Almost two decades ago, culture-independent molecular techniques were developed that identify bacteria directly from specimens through amplification, detection, classification, and quantification of bacterial DNA sequences rather than relying on cultivation [9, 10]. CF was one of the earliest disease models to which these new technologies were applied. Early reports identified complex microbial communities in CF sputum samples [11], abundant bacteria from the *Streptococcus milleri* group in sputum from adults with CF presenting with pulmonary exacerbations [12, 13], and high prevalence rates of anaerobes such as *Prevotella* in BALF from children with CF [14]. Since then, investigators have reported detection of over 1000 microbial species from CF respiratory samples [15, 16], and more than 500 papers have been published as of August 2020 with the keywords "cystic fibrosis" and "microbiome" (https://pubmed.ncbi.nlm.nih.gov). Airway microbiota in CF have also been shown to be distinct from microbiota detected in samples from children with other chronic respiratory diseases and healthy children [17–20], although core microbiota may overlap among children with other chronic respiratory conditions [21].

Culture-independent microbiological techniques applied to CF airway samples have advanced and diversified, now including next-generation sequencing, metagenomics, transcriptomics, metabolomics, and resistome profiling [22, 23]. Microbiota detection has expanded beyond bacterial pathogens to include viruses (virome), fungi (mycobiome), and nontuberculous mycobacteria [24–26]. In addition to respiratory disease, CF is also characterized by significant nutritional, GI, and hepatic complications. There is likely a strong interaction between respiratory and GI microbiome, immune development, and progression of lung disease beginning early in life (Fig. 6.1) [27]. Investigators have sought to understand the impacts of CFTR dysfunction, comorbidities, and therapies (particularly antibiotics) on the GI microbiome and associated clinical outcomes.

In this review, we highlight the current understanding of the constituents, dynamics, and clinical associations of the CF respiratory and GI microbiomes. We discuss respiratory sampling, microbial profiling techniques, and research approaches for airway and GI microbiota specific to CF. We also review what investigators have discovered over the last two decades about the relationships between microbial

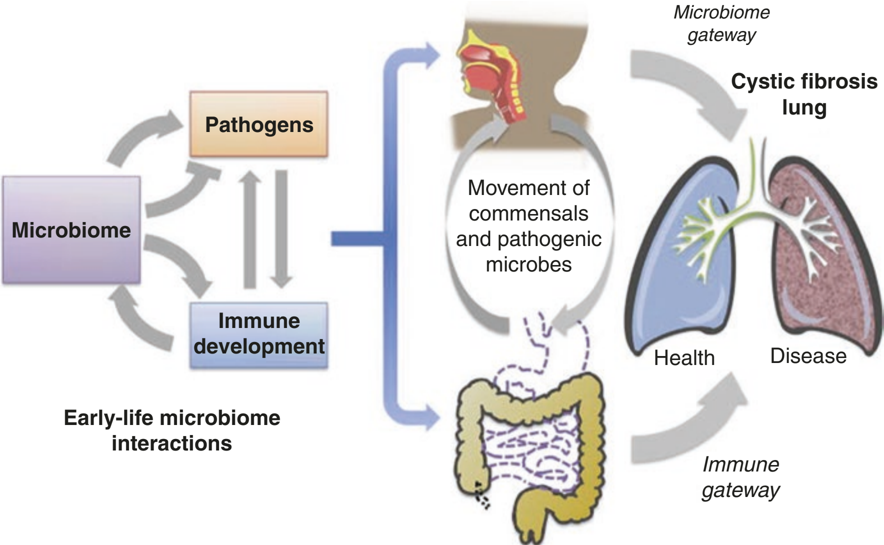

Fig. 6.1 Schematic of microbiome, immune development and pathogens in cystic fibrosis. Beginning in early life, the respiratory tract and gut microbial populations likely interact and develop microbiome characteristics protective against or permissive for pathogens. In the respiratory tract, the interplay between microbiota, pathogens, and immune response lead to airways injury and progression of lung disease. In the gastrointestinal tract, microbes may influence malabsorption and growth, intestinal health and function, liver disease, and systemic immune response. (Adapted with permission from Segal and Blaser [27])

community profiles and age, disease severity, and pulmonary exacerbations. Airway inflammation is a key driver of lung disease progression in CF [28]; thus, the relationship between microbiota and inflammation is highlighted. While this review focuses primarily on bacterial microbiomes, viruses and fungi are also common in CF airways and are potentially important components; studies of the virome and mycobiome are further expanding our understanding of airway microbial communities. We also highlight unique challenges in studying the CF GI microbiome, as well as clinical correlations and the impact of CF therapies.

Recently, CF care has been altered dramatically by the development and regulatory approval of CFTR modulators, small molecule therapeutics that partially restore CFTR activity for those with specific gene mutations [29]. CFTR modulators are now available for over 90% of the CF population in the USA beginning at 6 years of age and for some children as young as 6 months [30]. These relatively new therapies have had a dramatic effect on respiratory health in those who use them, and we summarize the current understanding of how modulators impact the airway microbiota. We end by summarizing key research questions that may help move findings from microbiome analyses from the research bench to the clinic to improve care of individuals with CF.

Respiratory Microbiome

Respiratory Tract Anatomy and Respiratory Samples

Any discussion of the CF respiratory microbiome is complicated by the fact that CFTR is expressed by epithelial cells throughout the airway. Accordingly, resulting mucosal pathology and alterations in normal mucosal microbiology can occur variably at different sites, including the sinuses, nasopharynx, oropharynx, central airways, and, more distal, increasingly small-diameter airways. CF respiratory microbiome work usually focuses on lower airway microbiology, most often by collecting sputum samples; however, directly sampling this distal space is difficult, particularly among children and people who do not reliably expectorate. Consequently, and because the CF upper airway is the site of many early respiratory symptoms, some CF microbiome research has made use of other sample types, including nasopharyngeal (NP) or OP swabs. Other studies have used more direct lower airway or lung sampling methods, including BALF, aspirates, or tissue samples. This variability in sampling types has complicated the construction of consistent models of CF respiratory microbiological dynamics and resulted in considerable controversy.

Sputum is often considered the ideal sample type for studying CF lung microbes, balancing convenience and accuracy for reflecting lower respiratory tract microbiology. However, studies scrutinizing this accuracy, including among people with pneumonia, identified variable contributions of oropharyngeal microbes, cells, and secretions to sputum samples [31]. To minimize, quantify, and control for these issues, researchers have used several strategies. For example, experimental assessment of the "quality" of a sputum sample has often involved examining for relatively high ratios of squamous epithelial cells (which indicates a high contribution of the upper airway) to polymorphonuclear cells (which may indicate lower respiratory infection), a schema supported by comparing the cultured microbes in paired sputa and transtracheal aspirates [32]. Hogan et al. compared the microbiota in sputum, BALF, and protected (bronchial) brush samples from the same people with CF, finding notable microbial profile differences in all three of these sample types [33]; similarly, Lu et al. observed significant differences in the microbiota of concurrent CF sputum and saliva samples [34], as did Prevaes et al., comparing NP, OP, and BALF samples [35], and Zemanick et al., comparing saliva, OP, and sputum samples [36]. Studies comparing directly sampled CF lung tissue, including from a lobectomy of a young child and from explanted lungs of people with end-stage lung disease, with contemporaneous lavages, swabs, and sputum, also identified differences in both culture and sequencing-based microbiota among these different sample types [14, 37, 38]. These results highlight the importance of considering the anatomic spaces that may be reflected by each sample type.

Contamination of Respiratory Samples

The issue of sampling lower airway microbiology of people with CF is increasingly complicated by the decreasing rates of expectoration that have been observed with improvements in prevention and treatment of CF lung disease, especially among children and with the advent of CFTR modulators [39, 40]. Inducing sputum production with inhaled hypertonic saline has been shown to be effective in many people incapable of spontaneous expectoration, and induced sputum cultures yield more "pathogens" than concurrent OP or upper airway swabs performed during coughing ("cough swabs") and contain microbiota similar to concurrent spontaneously expectorated sputum [36, 39, 41]. Therefore, sputum induction is a promising technique for sampling lower airway microbiology in the post-CFTR modulator era. However, even this sample type has limitations. Sputum must traverse many airway surfaces during expectoration, likely explaining differences often observed with concurrent, more direct lower airway samples as described above. Moreover, even serial-induced sputum samples from the same subjects frequently differ from each other, raising the possibility that different spaces in the lower respiratory tract are reflected by these samples at different times [42]. These sampling limitations impact nearly all CF respiratory microbiome analyses.

In addition to these anatomic challenges to accurately sample CF lower respiratory microbiology, sequencing-based studies must also consider the impact of microbial cells and DNA in the reagents used for sampling itself (such as saline used for lavages and swabs) and for processing respiratory samples [43]. Jorth et al. found, using a detailed, multistep BAL procedure to study children with CF, that the contribution of such reagent contaminants during sampling was relatively large among children with low respiratory bacterial abundances not dominated by standard pathogens and who had relatively mild disease, consistent with findings in other respiratory diseases [44] and indicating how particularly difficult it is to study the microbial determinants of early or mild airway disease. To quantify and address these limitations, researchers have adopted several strategies that go beyond microscopically enumerating salivary and lower airway host cells. For example, Lu et al. quantified the contribution of salivary microbes to sputum microbiota by collecting concurrent saliva samples, enabling them to computationally remove the salivary contaminants [34].

Some researchers have taken an entirely different approach to studying the CF respiratory microbiome by focusing on what can be learned from upper airway swabs alone. For example, Bogaert et al. have shown that the NP microbiota evolve distinctly among infants with CF compared to those without CF, and the researchers were able to characterize the impact of antibiotics on this relatively easily sampled space [45].

Microbial Profiling Techniques

As with sampling technique, microbiota profiling approaches have evolved substantially over recent decades. Among the earliest studies referred to as "microbiota profiling" of CF respiratory samples (and, indeed, of any respiratory samples) were published by the Bruce laboratory at King's College, London, beginning in 2003 using a gel electrophoretic method to profile 16S rRNA amplicons of adult CF sputum [11]. Since then, sequencing-based methods and quantitative PCR have largely supplanted these profiling approaches, with a variety of sequencing techniques represented, including pyrosequencing [46] and Illumina-based sequencing of 16S rRNA amplicons [47] and, more recently, metagenomic analyses [48, 49].

While 16S-based methods are very effective for measuring identities and relative abundances of most taxa in respiratory sample microbiota, metagenomic (shotgun) sequencing offers the opportunity to directly analyze predicted microbial community functions, in addition to taxonomic constituencies. Metagenomic sequencing also offers the advantages over amplicon-based methods of higher taxonomic resolution, strain-level analyses of individual taxa, and the potential for analyzing both host and nonbacterial microbial constituents (such as viruses and fungi). Therefore, with the expanding availability and decreasing cost of metagenomic sequencing, this approach seems likely to become more common in the CF respiratory microbiome space. However, these changes in methodology bring additional challenges to the field; for example, CF sputum often has relatively high ratios of human to microbial DNA due to abundant host inflammatory cells, limiting sequencing coverage of the microbiota, and investigators have addressed this issue in different ways [15, 44, 50, 51]. Similarly, different sequencing platforms and bioinformatic pipelines can substantially impact microbiota profiling results [52, 53]. Therefore, comparing or combining results from different studies can be complicated by methodologic variation and associated differences in bias.

Recent Developments in CF Respiratory Microbiome Research Approaches

To complement, confirm, and contextualize the findings from sequencing-based CF respiratory microbiome studies, researchers are also increasingly analyzing the microbial proteomic [54], metabolomic [55], and transcriptional profiles [56] of CF samples. Analytical approaches of microbiota data have also expanded and evolved, including consideration of the effect of co-occurrence of different taxa on clinical outcomes and microbiota composition [57] and, more recently, using sophisticated computational approaches to account for variation in methodology to allow for rigorous metanalyses [58]. In addition to increasing overall sample sizes and improving discriminatory power, these ancillary methods hold promise for moving away from simply cataloguing the constituents of CF respiratory samples to reflecting the

behaviors, and perhaps mechanisms of pathogenesis, persistence, and resilience, of the microbiota.

Variability Across Samples

CF lung disease is characterized by intermittent exacerbations of symptoms and progressive lung function decline over time and requires treatment with complex regimens of daily and intermittent medications, including antibiotics. Many of these aspects of the CF phenotype are associated with common patterns and temporal dynamics of airway bacterial community structures defined both within individual patients and between patients in the larger CF population. These clinical and treatment variations are thus relevant confounding variables for consideration in studies of CF respiratory microbiota. As changes in respiratory microbiota observed with advancing age and lung disease stage, as well as with pulmonary exacerbation, are covered in subsequent sections, this section focuses on within-patient variation during clinical change and with antibiotic use. The degree of variation between patients in CF respiratory microbiota has been consistently demonstrated to exceed that observed within patients, emphasizing the strength of longitudinal sampling to identify within-patient changes associated with outcomes of interest. However, within-patient variation in respiratory microbiota is present even when sampled repeatedly at clinical baseline (i.e., periods of clinical wellness, when episodic antibiotics are not being used). Analyses of daily sputum samples recently demonstrated within-patient variation in respiratory microbiota that exceeds the variation among DNA sequencing controls [59]. Thus, even at baseline clinical state, a single sputum sample may be limited in its representation of the respiratory microbiome of that patient over the broader time frame, again highlighting the strength of study designs that incorporate serial sampling.

Antibiotic use in people with CF can broadly be categorized as either episodic or chronic. Episodic antibiotics are inhaled, oral, and/or intravenous antibiotics, most often used to treat a pulmonary exacerbation. Chronic antibiotics include inhaled (typically alternating every other month) and oral (e.g., three times weekly azithromycin) antibiotics. Controlling for antibiotic use is relevant to both long- and short-term changes in CF respiratory microbiota at all ages. For example, chronic antibiotic use (i.e., antistaphylococcal prophylaxis) is associated with decreased bacterial community diversity in lower airway samples from infants with CF [60]. Research has demonstrated that cumulative antibiotic use over time is the primary driver of the decrease in bacterial community diversity, and the corresponding increase in relative abundance of CF pathogens, observed with advancing age and lung disease stage [61, 62]. Similarly, at a population level, episodic antibiotic use (i.e., samples collected while on antibiotic treatment for pulmonary exacerbation) is associated with decreased bacterial community diversity compared to samples collected at baseline clinical state [63].

Changes in chronic antibiotic regimens are similarly associated with variation in respiratory microbiota within patients. In a recent study of daily sputum sampling from six adults with CF during periods of baseline clinical state, bacterial community structures significantly shifted coincident with changes in chronic antibiotics (both inhaled antibiotics and changes in oral azithromycin use) [59]. Other recent studies of within-patient changes of respiratory microbiota following initiation of inhaled antibiotics have similarly demonstrated changes in bacterial community structures during a 28-day cycle of inhaled tobramycin [48, 64]. Of note, while inhaled tobramycin and aztreonam are both clinically indicated for chronic *P. aeruginosa* infection, the changes in respiratory microbiota observed with these inhaled therapies were not changes in *P. aeruginosa* abundance. Rather, inhaled tobramycin and aztreonam predominantly affected non-traditional taxa including obligate and facultative anaerobes, demonstrating "off-target" effects of inhaled antibiotics and emphasizing the relevance of chronic inhaled antibiotics as sources of variation in CF respiratory microbiota. Peleg et al. highlighted the importance of controlling for antibiotic use within patients in a study of respiratory microbiota following ivacaftor initiation [65]. In this study, ivacaftor treatment was associated with a decrease in sputum total bacterial load only in subjects with consistent antibiotic use across the study period. These data highlight the need to control for antibiotic use in studies of CF respiratory microbiota, as changes in respiratory microbiota resulting from antibiotic variation can mask other signals of interest.

Airway Microbiome Across Age and Disease Status

CF is a chronic, progressive disease that results in worsening airway injury and declining pulmonary function. Individuals experience different rates of progression (i.e., disease aggressiveness), with some developing severe lung disease in childhood and others entering adulthood with relatively preserved lung function [66, 67]. The reasons for this difference are not fully understood but likely include age of pathogen acquisition, environmental exposures, access to care, nutritional status, adherence to therapies, and underlying genetic modifiers. Given the progressive nature of the disease and variation in disease aggressiveness phenotype, studies of the CF airway microbiome need to account for both age and disease severity.

Microbial diversity has consistently been shown to decrease with age and disease severity in CF, with decreased richness and evenness primarily due to predominance of one or two primary bacterial pathogens (e.g., *P. aeruginosa, S. aureus*) with more severe lung disease and frequent use of antibiotics [58, 62, 68–71]. Coburn et al. studied airway samples from 269 individuals with CF ranging from 4 to 64 years using Si-Seq 16S rRNA gene analysis [68]. Alpha diversity was lowest in older patients and in those with more severe lung disease, defined by forced expiratory volume in 1 second (FEV_1). Core genera identified included *Streptococcus, Prevotella, Rothia, Veillonella,* and *Actinomyces*; CF pathogens including *Pseudomonas, Burkholderia, Stenotrophomonas,* and *Achromobacter* were detected

less frequently than these core taxa but tended to predominate when present, a finding also seen in other studies [72]. In a recent study, sputum samples from 299 patients across 13 CF centers in the EU and US were analyzed and results compared with clinical data [58]. Lower microbial diversity and increased dominance (defined by Berger-Parker dominance index) were associated with lower FEV_1 % predicted. CF pathogens, particularly *P. aeruginosa*, more often dominated respiratory microbiota in those with severe disease ($FEV_1 < 40\%$ predicted), whereas anaerobes dominated more frequently in those with milder lung disease. In another study, anaerobic bacteria cultured from CF sputum were again associated with milder disease, whereas cultivation of high quantities of *Pseudomonas* relative to anaerobes was associated with more severe disease, corroborating findings from sequencing analyses [73].

The CF lower airway microbiome has been investigated with 16S rRNA gene sequencing of BALF from infants, children, and adults [60, 74–77]. A cross-sectional study of BALF from children and adults from 13 CF centers in the USA found that *Streptococcus*, *Prevotella*, and *Veillonella* dominated the bacterial communities in young children and infants, while traditional CF pathogens predominated in those 6 years and older [75]. More diverse communities with mixed anaerobes had lower measures of airway inflammation (total cell count and neutrophils in BALF) compared to those dominated by CF pathogens. Conversely, Pittman et al. found that lower diversity was associated with reduced airway inflammation (neutrophil count and IL-8) in BALF from 32 infants with CF, possibly due to the use of anti-staphylococcus antibiotic prophylaxis within the study cohort [60]. In a study of infants and young children in Australia, 95 BALF samples were analyzed from 48 children, including 13 under 6 months of age [78]. Even within this young cohort, diversity decreased with age both on average for the entire population, as well as within longitudinally collected BALF from individual patients. Lower diversity was also associated with increased neutrophil elastase concentrations. The most prevalent genera were *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Neisseria*, *Haemophilus*, *Gemella*, *Graniculatella*, *Prevotella*, *Veillonella*, and *Streptomyces*. CF pathogen dominance within the community was associated with higher airway inflammation (IL-8 and neutrophil elastase). Diversity, including low diversity under 6 months of age, was not associated with lower lung function measurements at age 6 years. Finally, Laguna et al. analyzed 12 BALF samples from 8 infants with CF during clinical stability and found anaerobic bacteria in all samples [77]. Facultative and obligate anaerobes including *Streptococcus*, *Burkholderia*, *Prevotella*, *Porphyromonas*, and *Veillonella* were most abundant, with the CF pathogens *Pseudomonas*, *Staphylococcus*, *Haemophilus*, and *Stenotrophomonas* present in much lower abundance. Thus, interventions to prevent or delay the expansion and predominance of CF pathogens may be most effective in early childhood.

There is debate over the role of anaerobic infections and their true prevalence in the lower airways. Many of the anaerobes found in CF respiratory samples are present in oral microbiomes, and as discussed previously, whether the presence of these organisms in respiratory samples obtained through the oral cavity reflects lower airway infection or oral contamination is not always clear. Jorth et al. performed

BALF collection using procedures designed to minimize contact with oral secretions and contamination [43]. Contrary to the studies discussed above, they found low relative abundance of *Streptococcus*, *Prevotella*, and *Veillonella* in comparison to CF pathogens. Many of the genera detected in BALF were also detected in control samples from the bronchoscope, although most of these were not consistent with genera reported in previous investigations [17, 75, 77], and these BALF samples had notably lower total bacterial load. CF pathogens, but not anaerobes, were associated with increased airway neutrophil elastase. Other studies have also shown distinct communities when comparing upper airway (e.g., NP, OP, saliva) swabs to lower respiratory (BALF and lung tissue) sampling and relatively high bacterial loads in oropharyngeal and salivary samples [36, 38]. These studies highlight the importance of including reagent and bronchoscope control samples and minimizing oral contamination when possible when focusing on lower respiratory microbiology. Measuring associated airway inflammation also provides important information to estimate the impact of detected microbes on airway disease in CF.

While cross-sectional studies have provided key insights into CF respiratory microbial communities, longitudinal studies demonstrating changes over time within individuals and across populations during clinical stability and pulmonary exacerbations are critical for understanding the pathophysiology of disease. Ahmed et al. studied 241 OP swabs from 30 infants with CF collected over an average of 14 months [71]. *Streptococcus* and *Haemophilus* were the genera most commonly detected (55% and 12.5% of reads, respectively), and the presence of each was inversely related. *Staphylococcus* and *Pseudomonas* were rarely detected. Alpha (within sample) diversity did not change significantly with age; however, beta (between sample) diversity increased over time, reflecting shifting bacterial communities within individuals with age during infancy. As discussed in the section above, variability between samples must be considered in any longitudinal study, and further work is needed to understand the development of airway microbiota and relationship to disease progression [59]. Investigations of microbiome changes across periods of pulmonary exacerbation are discussed below.

Pulmonary Exacerbations

CF pulmonary exacerbations are broadly defined as periods of episodic worsening of CF lung health, consisting of a constellation of increased signs and symptoms such as cough, sputum production, and reduced lung function. CF exacerbations are treated largely with antibiotics and increased airway clearance and are generally assumed to be triggered by a disruption in one or more components of the mucus obstruction-infection-inflammation cycle that characterizes CF lung disease. Prior to culture-independent studies of CF respiratory microbiota, the bacterial triggers of CF exacerbations were predominantly thought to be increases in total respiratory bacterial burden and/or increases in abundances of known CF pathogens (e.g., *P. aeruginosa*). However, ~17% of CF exacerbations occur in the absence of detectable CF pathogens on bacterial culture [79], and these exacerbations respond

similarly to antibiotic treatment compared to exacerbations with identifiable CF pathogens. Using culture-independent analyses of CF airway samples, multiple studies have demonstrated that neither total bacterial load nor *P. aeruginosa* abundance (or relative abundance) increase with exacerbation onset [80]. Similarly, *P. aeruginosa* does not consistently decrease with antibiotic treatment for pulmonary exacerbation (including antibiotics directed against *P. aeruginosa*) [81].

Culture-independent studies of CF respiratory samples have thus investigated additional bacterial community characteristics as potential contributors to pulmonary exacerbations. As discussed above, the earliest culture-independent studies of CF airway samples found obligate and facultative anaerobes not typically identified with the selective bacterial culture performed in clinical microbiology labs to be prevalent in CF airways [14, 82]. Subsequent studies investigated the potential roles these anaerobes and other non-traditional CF species may play in pulmonary exacerbations. In early work, respiratory abundances of members of the *Streptococcus milleri* group were found to be associated with ~40% of CF exacerbations [12, 83]. Other anaerobes (e.g., *Gemella*) have also been associated with pulmonary exacerbation onset [84]. Other studies, however, have not identified changes in CF respiratory microbiota associated with pulmonary exacerbations at a population level [68, 75]. These seemingly discordant results may be at least partially explained by heterogeneity in CF respiratory microbiota across disease stage. For example, in a large study of 631 sputum samples from 111 CF patients, both bacterial community diversity and the summed relative abundance of the most common anaerobes increased during pulmonary exacerbation; however, this effect was only seen in patients in the early and intermediate stages of lung disease in which anaerobes are most abundant [63].

In addition to increases in bacterial community diversity and relative abundances of certain anaerobic species, bacterial community interactions and metabolic activities have also been associated with CF pulmonary exacerbations. Using an ecological networking approach of 16S rRNA gene sequencing data from CF sputum samples, Quinn et al. identified functional antagonism between anaerobes, *P. aeruginosa*, and *S. aureus* [85]. During times of clinical stability, these groupings were stable; however, co-occurrence patterns shifted in association with pulmonary exacerbation. Guttman et al. similarly identified shifts in bacterial community structures (including overrepresentation of anaerobes) and taxa interconnectedness associated with pulmonary exacerbation [86].

CF Respiratory Virome and Mycobiome

Microbiome investigations in CF have primarily focused on defining bacterial communities. More recently, investigators have applied molecular approaches to detect viruses (virome) and fungi (mycobiome) in CF respiratory samples [24, 26, 87]. Respiratory viruses, including influenza, respiratory syncytial virus, and rhinovirus, are known to contribute to pulmonary exacerbations in children and adults with CF [88–92]. Characterizing the virome presents unique challenges compared to the

bacterial microbiome. For example, optimal sampling approaches for virus detection may differ from those of bacteria. For viral detection, the upper airway, generally analyzed via nasal swabs or washes, provides more sensitive detection compared to oropharyngeal swabs [26]. While detection of viruses in sputum has been shown in some studies to yield similar results as nasopharyngeal samples, this approach is limited to those who are able to expectorate [93, 94]. Metagenomic approaches for virome analyses are challenging due to the relatively high genomic diversity of viruses, the lack of a conserved genetic region in viruses for taxonomic assignment analogous to the 16S rRNA for bacteria, and the relatively limited number of annotated viral genomes in databases [26]. Viral genetic material is also present in much lower quantities in respiratory samples than bacterial and human DNA, limiting sensitivity. There has been rapid development of multiplex PCR for detection of viruses using specific nucleic acid probes [95, 96], although this approach is limited to specific viruses. The recent development of commercially available PCR panels able to detect an increasingly broad range of viruses associated with human disease makes multiplex PCR a vital tool for viral detection.

Fungi are also commonly detected in CF respiratory samples, most commonly *Aspergillus fumigatus* and *Candida spp.* [97]. Fungi can be detected in microbiologic culture from CF airways, and the use of selective fungal culture media improves detection [98]. Approximately 5–15% of CF patients develop allergic bronchopulmonary aspergillosis (ABPA), an allergic inflammatory response to *Aspergillus* [more broadly, allergic bronchopulmonary mycosis (ABPM) when other fungal species are implicated] [99, 100]. However, culture detection of fungi in the absence of ABPA/ABPM has also been associated with worse respiratory quality of life scores and increased pulmonary exacerbations [101, 102]. Sequencing approaches have also been used to characterize the mycobiome in CF and other chronic lung diseases and may detect more complex fungal communities [24, 103, 104]. Mycobiome sequencing most frequently targets the internal transcribed spacers (ITS1 and ITS2) encoded between the 18S and 29S rRNA genes in fungi, although analysis can be challenging due to the relatively high sequence and length variability of this region [24]. As a result, few studies have employed this approach; one recent publication compared the respiratory mycobiomes of CF patients with and without pulmonary exacerbations, identifying likely interactions with bacteria of potential clinical importance [105]. Culture-based studies support this possibility; for example, coinfection with bacterial pathogens (e.g., *P. aeruginosa*) and fungi has been suggested to alter growth properties and virulence in both microbes, as well as resulting host responses; such inter-kingdom interactions remain important avenues of investigation [106, 107].

CFTR Modulators and Impact on Microbiome

With the approval of elexacaftor/tezacaftor/ivacaftor in 2019, more than 90% of people with CF will eventually be eligible for highly effective CFTR modulator therapy. By improving the functioning of the CFTR protein, CFTR modulators

improve mucociliary clearance and lung function and reduce pulmonary exacerbation frequency [108]. CFTR modulators are likely to have a positive impact on the natural history of CF respiratory infections through improvements in mucociliary clearance, preservation of lung function, and reductions in antibiotic use. However, given their recent introduction, data on the long-term effects of CFTR modulators on respiratory infections is fairly limited. Current data regarding CFTR modulators and impact on respiratory microbiome predominantly reflect experience with the first approved CFTR modulator, ivacaftor. Registry-based studies of patients on ivacaftor compared to contemporaneous controls have demonstrated associations between ivacaftor use and prevalence rates of *S. aureus*, *P. aeruginosa*, and *Aspergillus* [109–111]. Reduced prevalence rates of these CF pathogens consisted of both reduced infection acquisition as well as clearance of prior infection. Clearance of prior infections (e.g., *P. aeruginosa*) occurred in the minority (~30%) of patients, however, and was more likely to occur in patients with higher lung function, suggesting the CFTR modulators are likely to have more of an effect on CF respiratory microbiota in patients with earlier-stage lung disease. While less is known about other CFTR modulators and respiratory microbiota, a single-center study of patients on either ivacaftor or lumacaftor/ivacaftor found CFTR modulator use to be associated with delayed acquisition of CF pathogens, using a composite endpoint of *S. aureus* or *P. aeruginosa* infection [112]. These retrospective studies of clinical culture data are encouraging and suggest that CFTR modulators will have positive impacts on reducing rates of infection with known CF pathogens, particularly when modulator use is started early in the lung disease course.

Other culture-independent studies, however, have been less consistent in demonstrating sustained, positive impacts of CFTR modulators on CF respiratory microbiota. While certain studies have demonstrated reductions in relative abundances of CF pathogens and increases in bacterial community diversity (i.e., typically associated with milder lung disease) following modulator initiation [113], other studies have not identified increases in bacterial community diversity or reductions in CF pathogens or total bacterial load following modulator initiation [40, 108]. Many of these studies were limited by a short sampling period (e.g., 6 months) with a limited number of samples analyzed. In contrast, Hisert et al. performed long-term, serial sputum sampling of 12 adults with CF following ivacaftor initiation [114]. The eight patients with *P. aeruginosa* infection at ivacaftor initiation experienced a 60-fold decrease in *P. aeruginosa* abundance over the first weeks of therapy, an effect that persisted for the first 7 months. However, after a year of ivacaftor therapy, *P. aeruginosa* abundance had rebounded to pre-ivacaftor levels, and all of the eight patients remained infected with the same *P. aeruginosa* strains. Despite the rebound in *P. aeruginosa* abundance, clinical improvements related to CFTR modulator use were maintained across this time span and included improvements in FEV_1, reduced mucus plugging on computed tomography scans, and reductions in measures of airway inflammation. These data demonstrate that early changes in CF pathogen abundance related to CFTR modulator initiation may not be sustained over time (particularly in adults with established lung disease and infection) and are not necessarily reflective of clinical outcomes. As with the culture-based studies above, changes in respiratory microbiota with CFTR modulator use are heterogeneous

across the population, with greater changes seen in younger patients with earlier stage lung disease [40].

In addition to improving mucociliary clearance and lung function, CFTR modulators could also affect respiratory microbiota through direct antimicrobial activity and synergy with antibiotics. CFTR modulators in vitro have direct, dose-dependent effects on *S. aureus* and *P. aeruginosa* viability [115, 116], as well as synergistic activity with multiple antibiotics commonly used to treat CF respiratory infections, including vancomycin, trimethoprim sulfamethoxazole, moxifloxacin, linezolid, tobramycin, and ciprofloxacin [116–119]. Finally, CFTR modulator use has already impacted clinical monitoring of CF respiratory infection. Patients started on highly effective CFTR modulators commonly cease spontaneous sputum production, and this effect is likely to become more prevalent over time as the CF population benefits from highly effective CFTR modulators through lung function preservation. Induced sputum and OP swab sampling are likely to become more common for routine clinical monitoring, with consideration of BALF sampling in select cases in which a lower airways sample is needed to guide therapy (e.g., if sputum is not able to be obtained and nontuberculous mycobacterial infection is of concern) [120].

Antibiotic Susceptibility and Microbiome

CF pathogens including *P. aeruginosa* and other gram-negative bacteria frequently exhibit antimicrobial resistance based on standard microbiologic antimicrobial susceptibility testing (AST) [23]. Multidrug resistance is increasing in prevalence across the CF population [121] and is associated with worse outcomes [122–124]. Chronically infecting *S. aureus* lineages may develop bacterial-resistant adaptive mutants known as small colony variants, a slow-growing, difficult to treat phenotype that is associated with poorer outcomes in CF [125, 126]; methicillin-resistant *S. aureus* is also associated with worse patient outcomes [127]. Complicating management, standard AST may not reflect the response of bacteria within the CF airway to antimicrobials. In CF pulmonary exacerbations, antimicrobial treatment of *P. aeruginosa* or other gram-negative bacteria guided by AST does not improve clinical outcomes regardless of whether conventional AST [128, 129], combination or "synergy" AST testing [130], or biofilm resistance AST is used [131, 132]. Inhaled antibiotics used for management of chronic infections also reach higher concentrations than systemic delivery, likely overcoming some of the resistance seen in vitro.

Possible contributors to the discordance between antimicrobial susceptibility and clinical response are the presence of co-infections (with one or two other predominant microbes), the presence of complex microbial communities within the airways (including multiple bacterial, fungal, or viral species), and frequent, chronic exposure of the microbial community to antibiotics [23, 133]. With polymicrobial infections, mechanisms of altering antimicrobial susceptibility include antibiotic degradation, cell wall alterations, matrix component production that reduces antibiotic penetration, and metabolic alterations [133]. Anaerobes such as *Prevotella*,

often undetected by routine culture, frequently harbor resistance determinants, particularly when isolated from individuals with CF [134]. Even if non-pathogenic, *Prevotella* may release β-lactamase, conferring resistance to an otherwise susceptible co-infecting isolate of *P. aeruginosa* [135]. Cell wall disruption through lipopolysaccharide alterations has been demonstrated during co-infection with *P. aeruginosa* and *S. aureus,* leading to increased β-lactam resistance in *P. aeruginosa* [136]. Co-infection with *P. aeruginosa* and *S. aureus* has also been associated with emergence of small-colony variant *S. aureus* [137]. Metabolic products may be produced within the community which may alter growth and antimicrobial susceptibility [138–140].

Metagenomics has been used to identify resistance genes, or the *resistome*, within microbial airway communities in CF [141]. Lim et al. studied 10 sputum samples from 3 adults with CF during and after a pulmonary exacerbation. Using metagenomics along with microbiome and virome analyses, they identified many genes encoding resistance with substantial fluctuation in abundances over time. The virome also appeared to contribute to resistance genes, adding to the complexity. Allemann et al. analyzed the nasal resistome in 130 nasal swabs from 26 infants with CF during the first year of life. Antibiotic resistance genes (e.g., β-lactams or resistance to other classes of antibiotics) were detected in 53% of samples, including in samples obtained from infants with no prior exposure to antibiotics [142]. Most non-susceptible genes were associated with bacterial phyla rather than antibiotic treatment. Further studies of the CF resistome will help elucidate its relationship with microbial community constituencies, antibiotic treatment, and clinical outcomes.

Antibiotic resistance has been linked to lower respiratory microbial diversity [143]. This finding likely reflects the more severe disease phenotype seen in those with the multidrug resistance that results from higher lifetime antibiotic exposure. The presence of resistant bacteria also alters the impact of antibiotics on the microbiome. Hahn and colleagues found that changes in respiratory microbial diversity during and after pulmonary exacerbations treated with β-lactam antibiotics differed depending on whether plasma drug concentrations reached therapeutic concentrations [144]. Thus, while the makeup of the respiratory microbiome and virome impact the encoding and expression of antibiotic resistance determinants within the community, the resulting degree of resistance and antimicrobial dosing likely combine to influence treatment response.

GI Microbiome

The CF Gastrointestinal Microbiome

In contrast with respiratory samples, the microbiology of the CF GI tract has not been extensively studied. While the relatively intense historical focus on CF respiratory microbiology is likely due to the high morbidity and mortality attributed to CF

lung disease, there are ample reasons to examine the CF GI microbiome. For example, GI symptoms are often among the earliest and most severe manifestations of CF in children [145], and the GI microbiome is known to play key roles in the health, development, and function of the GI tract in general [146]. In addition, most people with CF have pancreatic exocrine insufficiency, leading to nutrient malabsorption and resulting in altered physicochemical conditions within the CF GI lumen compared to people without CF [147]. Therefore, the CF GI microbiome could be different from that of people without CF, and, if so, these differences could play a role in CF GI pathogenesis.

GI Microbiome Analytical Methods

As with the respiratory microbiome, researchers have used a variety of analytical approaches to study CF GI microbiology. Nearly all such studies to date have used fecal samples, an important sampling consideration because, similar to the discussion of sputum and its relationship with the lower respiratory tract above, these readily available specimens may not sample all anatomic spaces relevant for studying CF GI pathogenesis. For example, studies of GI tract mucosal health and microbial content of mouse [147] and ferret [148] models of CF have demonstrated differences in the small and large intestinal microbial content that may not be captured by studying feces. Similarly, and again reminiscent of the challenges in studying the CF respiratory tract, methods have varied over time within the CF fecal microbiome field, from gel electrophoretic methods [149] to array-based techniques [150], to 16S rRNA amplicon sequencing [151, 152], and more recently to metagenomic [153], proteomic [154], and metabolomic [155] approaches.

CF Fecal Dysbioses in Adults, Children, and Animals with CF

Despite this methodologic variation, GI microbiome studies have generally identified differences in fecal microbiology among people with versus without CF. These differences, commonly referred to for simplicity as "dysbioses" relative to the non-CF GI tract, depend on the study population (e.g., infants versus adults), as well as on important confounders, including diet, antibiotic exposure, and other treatments, including probiotics, acid blockers, laxatives, and CFTR modulators. Regardless, the studies to date have identified some general principles that provide clues to the dominant forces selecting for the fecal microbiota [156] and the role of these microbes in CF disease pathophysiology.

In infants, the CF fecal dysbiosis is characterized by relatively high abundances of *Proteobacteria*, including (but not limited to) *Escherichia coli* [151, 156, 157],

and relative depletions of a number of taxa with metabolic activities that are associated with immune and intestinal health [152, 158]. For example, many species in the phylum *Bacteroidetes* and others in the *Verrucomicrobia* have been shown to be relatively underrepresented in CF compared to non-CF infant fecal samples. These taxa are key producers of short-chain fatty acids (SCFAs), metabolites known to modulate mucosal inflammation, provide nutrients and calories to the host, and regulate intestinal development [152, 156, 159]. Consistent with these roles, studies have demonstrated significant relationships between the magnitude of the infant CF fecal dysbiosis and fecal measures of inflammation [152, 160, 161], as well as with risk of respiratory manifestations of CF such as exacerbations. The latter finding may relate to the observation that some taxa observed in CF infant fecal samples are subsequently identified in those infants' respiratory samples, perhaps indicating an important role for the GI tract in "seeding" the airways or, alternatively, for the immune-modulatory effects of the GI microbiota in airway inflammation (the "gut-lung axis") [162].

While the details differ from human children with CF, fecal and GI dysbioses are also observed in diverse CF animal models. These studies have been complicated by both sampling issues and variation in model. For example, mice with CFTR mutations raised under "conventional" conditions have profound bacterial overgrowth [163] of their small intestines that is not clearly reflected in fecal samples, and the fecal microbiota of such mice depend more on environment, mouse strain, and housing conditions than on CF itself [164]. Similar observations were made in CF ferrets [148]. However, when raised under specific pathogen-free conditions, the fecal microbiota of CF mice were uniformly different from those without CFTR mutations [165], strongly suggesting that CFTR dysfunction itself selects for different microbiota, consistent with observations in infants.

By contrast, studies of the adult CF fecal microbiome have revealed relatively similar impacts of CF itself and of the confounders described above, including diet, antibiotics, and age. For example, Fouhy et al. identified some similarities and some differences from the findings in infants when comparing fecal samples of adults with and without CF; specifically, *Bacteroidetes* relative abundances were relatively low, as seen in infant studies, while those of *Firmicutes* were relatively high, which differed from observations in infants [155]. However, the magnitude of these differences depended in part on the number of intravenous antibiotic courses received in the prior year [166]. These results have been supported by a CF infant microbiome study that demonstrated a significant effect of antibiotics on fecal microbial diversity, as well on as the abundances of health-associated taxa, including *Akkermansia, Bacteroides*, and *Bifidobacteria* [158]. This finding and the observation that diets differ substantially between people with and without CF [167] complicate the interpretation of studies demonstrating significant relationships between adult fecal microbiota, lung function [155] and CFTR genotype [168], all of which likely correlate with antibiotic load.

New Clinical Correlations with the CF Fecal Microbiome

Relationships between CF fecal microbiota and clinical outcomes have now been established beyond measures of inflammation and respiratory disease that provide some tantalizing and promising clues to CF disease pathogenesis beyond the intestine. For example, fecal microbiota metrics were found to correlate significantly with measures of both intestinal permeability and cirrhosis of the liver [169]; CF liver disease is a significant comorbidity impacting quality and length of life in people with CF. As the pathogenesis of CF liver disease has been hypothesized to relate to translocation of bacterial products into the portal circulation, these findings may indicate a role for the CF GI microbiota in the development of cirrhosis, as well as a potential route to preventing this complication. Similarly, recent studies suggested a role for the infant GI microbiome in developing endocrine function—specifically, the secretion of growth hormones that regulate bone and body growth. Analyses of germ-free and specific pathogen-free mouse models suggested that SCFAs, produced by GI microbes, stimulate the production of the insulin-like growth factor-1 (IGF-1), which regulates bone deposition and has been consistently shown to be depleted in people and animals with CF [170]. Subsequently, a recent study found a significant relationship between the magnitude of the infant CF fecal dysbiosis and linear growth failure [153], which is also known to correlate with serum IGF-1 [171]. Together, findings such as these highlight the potential for fecal microbiome studies to reveal unexpected, and perhaps surprising, new models of pathogenesis and resulting candidate treatment targets, with potential impacts beyond CF.

The Effects of CF Treatment on Fecal Microbiomes

Because features of CF fecal dysbioses correlate with various clinical outcomes (as described above), there is interest in studying both the effects of common, current CF therapies on GI microbiology (and, consequently, the potential role of those effects on clinical outcomes), as well as the clinical utility of modulating the CF microbiome with novel treatments. For example, antibiotics are known to significantly alter the CF fecal microbiome; as noted above, however, whether those effects play any direct, causative role in the clinical response to antibiotics is difficult to distinguish from indirect relationships [166]. In contrast, some clinical trials of oral probiotics have shown promise, albeit with relatively small effect sizes, with reported changes in fecal microbiota concurrent with decreased fecal measures of inflammation [172] and in exacerbation risk [173]. However, other studies have not consistently shown the same effects [174]. The CF fecal microbiota have been shown to be capable of producing SCFAs when provided with the right substrate (nonfermentable starch), raising the possibility that prebiotic approaches may be more effective than probiotics [175].

Perhaps the most convincing route to investigating the direct relationship between CFTR dysfunction and GI microbiology in people with CF is to examine the effects of CFTR modulators on fecal microbiomes and concurrent clinical measures. There is growing evidence that these medications have impacts on the GI tract, among other nonpulmonary tissues [176]. While multiple such studies, in different CF patient populations, are ongoing (Clinicaltrials.gov NCT04038047 and NCT04509050), to date one study in eight children and eight adults with CF reported decreased fecal measures of inflammation, and improvements in several taxonomic features of the CF fecal dysbiosis, with a median of 6 months of the highly effective CFTR modulator ivacaftor [177]. Therefore, the CF fecal dysbiosis may be at least partially reversible, alleviating at least some features of CF GI dysfunction, a hypothesis that awaits confirmation and mechanistic detail.

Remaining Challenges in CF Microbiome Studies

Over the past decades, novel microbiologic techniques have upended our understanding of chronic airway infection in CF [178, 179]. Once thought to be disease where one or two pathogens drove airway injury and disease progression, we now know that complex microbial communities exist as a continuum along the sinorespiratory tract. Pulmonary exacerbations, key events in individuals with CF associated with more rapid disease progression, are likely more complex in etiology than previously understood. Changes in respiratory bacterial communities before the onset of exacerbation and their response to antimicrobial therapy do not necessarily conform to the prevailing theories of disease pathophysiology. The CF GI tract also harbors a unique microbiome, and dysbiosis has repercussions for nutrition and growth, GI and hepatic outcomes, and systemic inflammatory response and respiratory outcomes in CF [180]. Table 6.1 highlights key research challenges in CF microbiome investigations.

Early investigations of the airway microbiome brought hope that identification of difficult to cultivate microbes, particularly anaerobes, would lead to rapid improvements in therapy. For example, if *Prevotella* was contributing to pulmonary exacerbations and hindering complete clinical recovery, then broadening antibiotic treatment to include anaerobic coverage would be hypothesized to improve outcomes. The actual progression of knowledge and translation to clinical care has proven more difficult for a number of reasons. First, limitations of sampling the contiguous respiratory tract, with its innate heterogeneity of microbial communities throughout the upper and lower respiratory tracts and within different lung compartments, have made it difficult to confirm the presence, and immunologic and pathologic roles, of nontraditional microbes in CF lung disease. Next, each of the diverse sequencing technologies used in this field introduces specific biases into resulting analyses, and these approaches can be hindered by lack of quantification, difficulty with species identification, and inability to distinguish active microbes from residual DNA products. Improved informatics now allows species-level identification for

Table 6.1 Key research challenges

Research challenges	
Specimen collection	Optimal sampling of lower respiratory and gastrointestinal tracts; minimizing contamination from other spaces, tools, and reagents
Optimizing sequencing technologies	Bacterial species identification, detection, and identification of viruses and fungi, accurate quantification of microbes, use of metagenomics and transcriptomics, managing human vs. microbial DNA, discriminating between active microbes and residual DNA
Understanding microbial interactions	Development of in vitro models of polymicrobial infections, accurately modeling human CF airways, recapitulating CF microbial communities
Progression in early life	Identifying a window of opportunity to delay or prevent chronic infection of known pathogens
Antibiotics	Impact of early antibiotics on respiratory and gastrointestinal microbiota; development of antimicrobial resistance; relationship of antibiotic pharmacokinetics and microbiota; modulation of the gastrointestinal microbiome to improve clinical outcomes
Bacteriophage	Use of bacteriophage for difficult to treat infections; profiling endogenous bacteriophage and their role in microbiota dynamics
CFTR modulation	Impact of modulators on microbiota in individuals with and without underlying structural lung disease and chronic airway infection
Understanding clinical impact	Understanding how disease and inflammation in the respiratory and gastrointestinal tracts relate to the microbiota and their changes

many bacteria, and metagenomics and transcriptomics are advancing our ability to distinguish bacterial activity with direct sampling. Thirdly, the role of microbes within communities is likely much more complex than our previous paradigm of pathogen → inflammation → airway damage or the treatment approach of antibiotics → pathogen killing → recovery. Bacterial-bacterial (and bacterial-viral and bacterial-fungal) interactions through quorum sensing, development of biofilms, geographic heterogeneity, immune stimulation, nutrient sharing, competition, and interplay of antimicrobial resistance mechanisms all contribute to a complex environment. Finally, many of the antibiotics used in CF are broad-spectrum and have altered pharmacokinetics in individuals with CF. These antibiotics may have unintended or unexpected effects on the microbial communities in the respiratory and GI tracts. Thus, our understanding of why certain antibiotics result in clinical improvement or not may be based on faulty assumptions.

One area in which microbiome studies have been particularly informative is early disease progression in infants and young children with CF [27]. Measures of airway inflammation are lower in those who do not harbor traditional CF pathogens such as *P. aeruginosa* and *S. aureus*, even when mixed anaerobic microbes are detected. Studies have also found a relationship between gastrointestinal and respiratory microbiota early in life [181]. Thus, early childhood represents a window of opportunity to prevent development of infections and to potentially modulate the microbial communities to make them more resilient to pathogen invasion.

With the availability of CFTR modulators, changes in the prevalence of airway pathogens is likely to change. This development represents a remarkable opportunity to study changes in the airway and GI microbiome in populations newly initiating modulator therapy. The impact of therapy on microbial communities is likely to differ between individuals with moderate to severe bronchiectasis compared to those in whom bronchiectasis has not yet developed. Understanding these changes will be critical for future antimicrobial recommendations.

Finally, better understanding the roles of bacteriophages in the CF respiratory microbiome offers the potential for novel therapeutic approaches. Metagenomic studies of CF respiratory samples have identified the presence of diverse bacteriophages in the CF lungs [182, 183]. Transitions between lytic and temperate dynamics of these endogenous phages likely contribute to modulation of bacterial community structures and CF lung disease [184]. For example, Pf bacteriophages have been identified in ~40% of people with CF and *P. aeruginosa* infection. In addition to regulating *P. aeruginosa* density, Pf phage can alter *P. aeruginosa* virulence in vitro by modulating *P. aeruginosa* biofilm formation and antibiotic resistance and in vivo by impeding host innate immune defense [185, 186]. Development of therapies or vaccinations to target endogenous phages to prevent or reduce virulence of *P. aeruginosa* and other CF pathogens is a potential future novel approach to treating CF infections. Successful use of bacteria-specific lytic phages to treat CF infections has been reported in a small number of cases [187–190], and larger clinical trials of phage therapy for treatment of multi-drug resistant CF infections are currently underway.

Conclusions

Tremendous advances have been made in understanding CF microbiomes and their contribution to disease. While the translation of these findings to improved therapeutics has proven more difficult than initially hoped, future research has the potential to provide novel insights into the pathophysiology of disease and to transform treatment approaches for individuals with CF.

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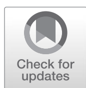
Chapter 7
Bronchiectasis



Micheál Mac Aogáin, James D. Chalmers, and Sanjay H. Chotirmall

Introduction

Aetiopathogenesis

Bronchiectasis is characterized by chronic, irreversible dilatation of the bronchi with thickening of the airway walls linked to degradation of bronchial elastin and supportive tissue structures. Patients experience chronic cough and recurrent respiratory infections associated with pulmonary exacerbations, and increased inflammation, that leads to airway damage, dyspnea and lung function decline [1, 2]. The gold standard for its confirmatory diagnosis is high-resolution tomography (HRCT) which can further delineate morphological subtypes. Bronchiectasis can be cylindrical, common and characterized by smooth tubular bronchi and mild disease; varicose, non-uniform dilation; or cystic, associated with more severe disease and complete loss of bronchial morphology [2, 3]. While the largest airways become visibly dilated, patients exhibit airflow limitation due to impaired drainage of bronchial secretions and obstruction in the small and medium airways caused largely by

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infectious and inflammatory insults [2, 4, 5]. Mucus inspissation and impaired mucociliary clearance, among other factors, support microbial colonization of the lung, a central tenant of current aetiopathogenic models [4, 6]. Clinically, patients present with cough and chronic sputum production, antecedent to confirmatory HRCT diagnosis, while other associated symptoms include malaise, chest discomfort, haemoptysis and weight loss [1, 2, 7]. Post-infectious bronchiectasis represents a key aetiology (after idiopathic disease) followed by immunodeficiency, ciliary disorders and obstructive lung disease, although estimates of each vary internationally [2, 8]. The vicious cycle hypothesis, first described by Cole, proposes that trigger factors, underpinned by genetic susceptibility or defects in host defence, set in motion a self-perpetuating cycle of infection, inflammation and impaired mucociliary clearance leading to progressive bronchial wall dilatation and destruction [4]. Paediatric bronchiectasis exhibits a distinct presentation compared to adults with a predominance of specific aetiologies and clinical manifestations including primary and secondary immunodeficiency, ciliary dyskinesia, congenital malformations, bronchiolitis obliterans and skeletal disease [8]. Airway insults from recurrent childhood infection further predisposes to the development of bronchiectasis [9]. It is noteworthy that the occurrence of bronchiectasis peaks at the extremes of age (i.e. in children 75 years) as these life stages are accompanied by significant shifts in both the microbiome and underlying immune status, which, in turn, may influence disease trajectory while providing scope for intervention [5, 10, 11].

The Role of Infection

Infection is a hallmark of bronchiectasis as both a cause and consequence of disease. Culture-based studies have played a crucial role in our understanding of the microbiology of the bronchiectatic airway, where *Haemophilus influenzae, Pseudomonas aeruginosa, Streptococcus pneumoniae, Moraxella catarrhalis* and *Staphylococcus aureus* are frequently identified [2, 8, 12, 13]. *Mycobacterium tuberculosis* is also implicated as an important post-infective aetiology, particularly in Asia [8, 14], while infection by non-tuberculous mycobacteria (NTM) is associated with a worsening of pre-existing bronchiectasis and increased risk of fungal colonization by *Aspergillus fumigatus* [15–17]. Fungi are also important aetiological agents with heightened sensitization and the development of allergic bronchopulmonary aspergillosis (ABPA) increasingly recognized as negative prognostic indicators, while fungal genera including *Candida*, *Penicillium*, *Cryptococcus*, *Clavispora* and *Scedosporium* have been highlighted in more recent culture-independent studies of the bronchiectasis airway [8, 17–20]. The precise role of viruses in bronchiectasis is not well-established, with few large-scale and prospective studies available; however, several established respiratory viruses including coronavirus, rhinovirus and influenza have been commonly detected in bronchiectasis patients [2, 8, 21, 22]. While increasing evidence also supports a role for the human T-cell lymphotropic virus type 1 (HTLV-1) in acquired immunodeficiency

linked to disease risk, conclusive mechanistic studies are lacking [8, 23]. Likewise, oropharyngeal species – generally reported as 'contaminant' microbes – may play insidious 'pathobiont' roles inciting deleterious immune responses directly or through their influence on overtly pathogenic species through microbial interactions [24, 25]. As such, the bronchiectasis microbiome may be best defined as a dynamic inter-kingdom network with an underlying and dysregulated production of cytokines, elastases and matrix metalloproteinases (MMPs) that, in turn, damage the structural integrity of the lung, leading to visible distortion of the airway [26–28]. Inciting microbial insults elicit a largely neutrophilic cellular immune response with increased macrophage recruitment, while a small but significant subset of patients exhibit eosinophil-dominant disease linked predominantly to environmental triggers [29]. The observed cytokine responses are heterogenous and reflect the nature of the underlying infectious triggers within the bronchiectasis airway and are typically characterized by elevated levels of IL-1β, IL-8, leukotriene (LT)B_4, CXCL2 and TNFα [29]. These inflammatory profiles sustain the release of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) from the endothelium, leading to further neutrophil and eosinophil recruitment to the airways [29, 30]. The role of the neutrophil remains central to bronchiectasis and has been elegantly dissected in several studies which collectively illustrate its importance in the pathogenic process. The neutrophil, as a major airway inflammatory cell, produces serine proteases such as neutrophil elastase, which itself is described to represent a negative prognostic indicator in bronchiectasis which correlates to reduced microbial diversity and the presence of *P. aeruginosa*. Specifically, neutrophil elastase is highly expressed in bronchiectasis and associates with exacerbations, radiological extent of disease and lung function [26]. While active in bronchiectasis, the neutrophil itself is functionally compromised, leading to impaired bacterial phagocytosis and killing despite a prolonged viability and delayed apoptosis [31, 32]. Alteration of the sputum proteome in bronchiectasis patients infected by *P. aeruginosa* reveals the upregulation of pregnancy zone protein (PZP) associated release of neutrophil extracellular traps (NETs) tying airway infection to NET formation, disease severity and pathogenesis [33, 34]. Such detailed mechanistic study of the neutrophil has paved the way for clinical application in bronchiectasis evidenced by the success of a phase II clinical trial of the dipeptidyl peptidase 1 inhibitor (DPP-1) Brensocatib – an inhibitor of neutrophilic serine protease activation. This opens a new and urgently needed avenue towards clinical translation [35, 36]. The dysregulation of host neutrophilic function in bronchiectasis is also notable for its association with shifts in microbiome composition, whereby neutrophil-associated PZP levels predict a dysbiotic predominance of Proteobacteria including *Pseudomonas*, Enterobacteriaceae, *Stenotrophomonas* and *Moraxella* further illustrating the close relationships between microbiome profile and disease pathogenesis [33]. This particular association has been independently corroborated in subsequent microbiome studies and correlated to neutrophil elastase levels, which in turn associated with a decreased microbial diversity and increased *P. aeruginosa* abundance [37]. The salient features of bronchiectasis pathogenesis as currently understood are detailed in Fig. 7.1.

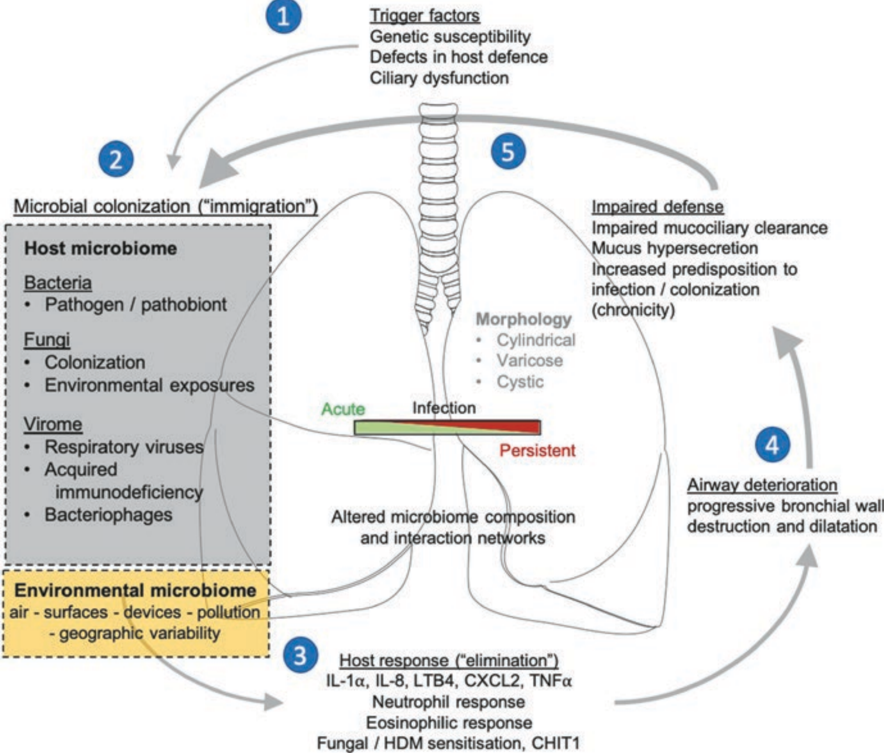

Fig. 7.1 Overview of the pathogenesis in bronchiectasis and the role of the microbiome. (1) Trigger factors include host genetics, defects in host defence and/or ciliary dysfunction which predispose to microbial colonization. (2) Colonization or net 'immigration' of microbes leading to the emergence of deleterious microbiome signatures defined by the presence of host response to specific bacteria, fungi and viruses (grey box). Environmental exposures to air-, surface- and device-associated microbes including pollution, and other geographically variable environmental factors may also contribute (yellow box). (3) Deleterious and impaired host responses directed towards the 'elimination' of microbes are triggered by pathogen-associated molecular patterns, virulence factors and allergens culminating in a dysfunctional and airway damaging immune response. A transition from acute to persistent infection is accompanied by a decreased diversity in the microbiome, altered composition and network configuration. (4) Chronic infection and inflammation cycles, leading to progressive airway damage and loss of structure with associated changes in radiological morphology. (5) Loss of structure and increasing dilatation of the bronchi, coupled to mucus plugging and impaired mucociliary clearance predisposing to an increased risk of subsequent infection and progressive clinical decline

The Case for Microbiome Research in Bronchiectasis

Building upon Cole's initial hypothesis, a more complex and holistic picture has emerged as sophisticated multi-omic technologies are increasingly applied in bronchiectasis and other chronic respiratory diseases providing both opportunities and

challenges [38]. Fine-structure analysis of disease progression permits a greater appreciation of pathogenic mechanisms, while improved patient stratification provides scope for personalized therapeutic approaches [36, 39]. Contemporary models such as the recently advanced 'vicious vortex' paradigm proposed by Flume et al. seek to capture the dynamic interactions embodied by each pathophysiologic step of the disease cycle as they promote persistent and progressive inflammation and airway damage over time [6]. This represents a major academic and clinical challenge in the setting of a highly heterogenous condition such as bronchiectasis, where arrival at the endpoint of structural airway damage may be reached through distinct endophenotypic routes [36, 39]. In this context, the microbiome represents a potentially important window into disease progression and pathogenesis with prognostic potential and scope for improved patient stratification in this heterogenous clinical setting (Fig. 7.1) [36, 40, 41]. The failure of high-profile multi-centre antibiotic clinical trials in bronchiectasis has further highlighted the need and potential for the integration of microbiome approaches into clinical trial design with the aim of improved patient selection and stratification including appropriately chosen clinical endpoints that address past inconsistencies resulting in a failure to replicate results across geographically distinct patient populations [8, 36, 42]. Early work, driven by culture-based assessment of resident microbial pathogens and later advanced by characterization of associated immune responses, has laid the foundation for our understanding of bronchiectasis and the potential therapeutic approaches for its clinical management [12]. Intriguingly, however, the introduction of antibiotics for bronchiectasis-associated microbiomes has not been met with the anticipated decline of this 'infective' condition, contradicting a simplistic model of bacterial overgrowth amenable to suppression by antibiotics. Therefore, early presumptions regarding amenability to antimicrobials to provide resolution has likely contributed to a neglect of this complex and poorly understood respiratory condition [2, 36]. A further feature contributing to delays in understanding the airway microbiome is the previous and incorrect assumption of lung sterility. As a consequence, the lung microbiome has received less attention compared to other anatomical sites (such as the gut) leading to significant gaps in understanding the lung microbiome – gaps now actively being closed in the context of data illustrating the presence and key functional role of the microbiome in the respiratory diseases including bronchiectasis [38, 43]. The time for a more detailed exploration of the microbiome in bronchiectasis has arrived and will likely provide a clearer understanding of disease pathogenesis, mechanisms of infection and a potential for therapeutic advancements in its management.

The Airway Microbiology in Bronchiectasis

Several studies have described airway colonization in bronchiectasis by distinct microbial entities, largely based on culture. Although prevalence varies, *H. influenzae* and *P. aeruginosa* represent the most common bacterial species identified

followed by *S. pneumoniae, M. catarrhalis, S. aureus* and others including *K. pneumoniae*, while *S. maltophilia* and *Achromobacter xylosoxidans* are less frequently found [13, 44]. While extensively characterized, a significant bias exists towards western populations in the microbiological surveillance of the bronchiectasis airway, and the growing awareness of geographic differences most notably in Asian populations have been described warranting study [8, 45]. In comparison to western populations, Asian patients exhibit higher *P. aeruginosa* colonization rates (relative to *H. influenzae*), while *K. pneumoniae* is also more frequently isolated [45]. A comparative analysis of European, US and Indian registry data also reveal similar patterns with higher rates of *P. aeruginosa* and exacerbation risk associated with prior *M. tuberculosis* infection in Indians [14]. Given such disparities, other geographic and population-associated differences in microbiology and microbiome composition may exist contributing further to disease heterogeneity and therapeutic challenges [2, 8, 36]. Importantly, no microbial pathogen has been identified in up to 70% of bronchiectasis sputum cultures – even in the presence of clear and measurable inflammatory responses – further highlighting our incomplete understanding of pathogenesis and, perhaps importantly, the need for broader microbiome analysis unrestricted by selective culture-based methodologies [29, 41, 46]. Genetic analyses of the microbiome, aided by next-generation sequencing (NGS), are now emerging in bronchiectasis, supporting the extensive culture-based literature (Fig. 7.2). The derivation and interrogation of such data may ultimately allow for an integration of direct microbiome sequencing into clinical diagnostics and the selection of therapies, potentially offering more personalized and effective treatment approaches at the individual level in the future care of bronchiectasis.

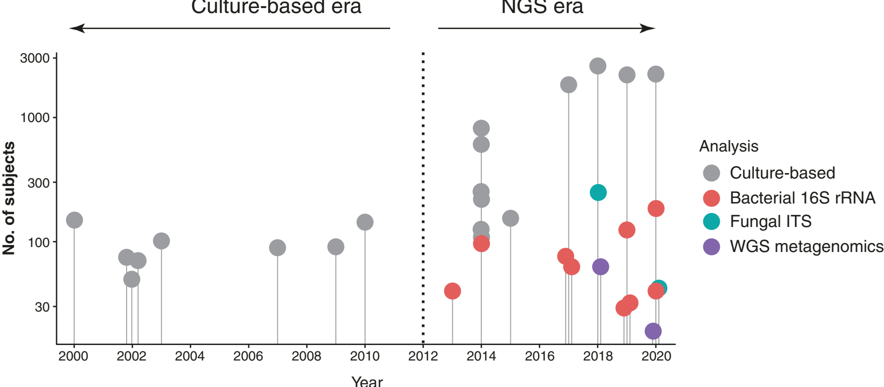

Fig. 7.2 Timeline of culture-based and culture-independent microbiome research in bronchiectasis. A lollipop chart illustrates the growth of culture-based and culture-independent research on the bronchiectasis microbiome over time (2000–2020). Studies are indicated by coloured lollipops with stick height (y-axis, logarithmic) representing the number patients in each study. The type of analysis performed in each study is indicated in the right-hand legend (grey, culture based; red, bacteria 16S rRNA analysis; blue, fungal ITS analysis; purple, WGS metagenomic shotgun analysis)

The Bacteriome in Bronchiectasis

While the emergence of culture-independent lung microbiome analysis has its origins in cystic fibrosis (CF), it is only more recently that culture-independent approaches have been applied to (non-CF) bronchiectasis (Fig. 7.2) [41, 47]. Pioneering pyrosequencing efforts initially documented a limited shift in community composition during exacerbation and following antibiotic therapy with characterization of *P. aeruginosa*, *H. influenzae, Prevotella* and *Veillonella* as part of a complex community in a cross-sectional cohort of 40 patients [48]. Rogers et al. subsequently applied 16S rRNA sequencing to a larger cohort (n = 86) from a clinical trial of macrolide intervention (the BLESS study) highlighting this as a potentially informative analytical measure in respiratory trials [49]. The BLESS intervention (low-dose erythromycin – 400 mg twice daily) demonstrated success in reducing exacerbations among longitudinally sampled patients compared to the control arm with 16S rRNA analysis providing insight into microbiome composition as a correlate of the observed therapeutic response. This inclusion of microbiome analysis revealed granular changes in microbiome composition and allowed patient stratification according to the dominant organism, where a significantly worse outcome is observed in those with *Pseudomonas* or *Veillonella*-dominant profiles [40, 49]. The association of the commensal genus *Veillonella* with exacerbation further represents a novel perspective on this bacterial taxa and suggests a potential insidious role for this anaerobe within the complex bronchiectasis microbial community [40]. Microbiome profiles importantly were also predictive of the observed host immune response, with *H. influenzae* inducing increases in MMP2 and MMP8 compared to patients with *Pseudomonas*-dominant profiles, while both organisms induced significantly elevated levels of serum CRP, sputum IL-1β and IL-8. Taxonomic diversity exhibits negative correlation with clinical outcomes, and lower IL-1β and IL-8 for instance suggest better outcomes with greater microbial richness observed in the erythromycin treatment arm [28, 49]. In further corroboration of the observed microbial-host interactions in the BLESS cohort, loss-of-function variants are identified in the human FUT2 fucosyltransferase gene (responsible for coating mucosal surfaces with fucosylated glycans) and found to influence the composition of the microbiome while decreasing the risk of pulmonary exacerbations and *P. aeruginosa* colonization [24]. Overall, the targeted 16S analyses of the BLESS cohort underscored the stability of the bronchiectasis microbiome but noted the supplantation of *H. influenzae* by the more pathogenic *P. aeruginosa* strains. Such displacement, along with the observed increases in macrolide resistance, confers potentially undesirable long-term consequences of this therapeutic intervention, given the established negative association of *P. aeruginosa* with bronchiectasis [44]. Therefore, while the effect of erythromycin therapy is largely beneficial, this depends on the baseline microbiome composition and importantly did not significantly alter exacerbation rates in *Pseudomonas*-dominant patients, while its benefits in non-*Pseudomonas*-dominant patients came with a greater risk of subsequent *Pseudomonas* colonization. Further analysis, based on whole-genome

metagenomic shotgun sequencing, has also highlighted the increased burden of macrolide resistance determinants in antibiotic-treated patients over time [50]. These observations clearly highlight the value of targeted and metagenomic sequencing of the microbiome as a key secondary outcome measure in clinical trials and administered therapeutics. A similar trend in antimicrobial resistance, through metagenomics, was observed in macrolide-treated severe asthma, further underscoring this phenomena in chronic respiratory disease [51]. Additional ultra-deep metagenomic analysis of respiratory specimens from non-diseased (healthy) subjects reveals the presence of a core macrolide resistome, which importantly remains consistent even across distinct respiratory disease states, including bronchiectasis [52]. Resistance genes are correlated to commensal species representing potential resistance reservoirs, detected in host sputum and on patient inhaler devices highlighting the potential of metagenomics for surveillance of resistance in the environment as well as within host respiratory microbiomes [52].

While baseline microbiomes appear predictive of clinical course and therapeutic response, stability throughout exacerbation and treatment has emerged as a recurrent finding among microbiome studies in bronchiectasis [41, 49, 53]. Indeed, individual microbiomes can persist for many years in longitudinally sampled patients [54]. There is, however, a generally consistent correlation between diversity measures of the microbiome and clinical outcome that likely reflects the increased dominance of particular pathogens, in agreement with the adapted island model of the lung microbiome, where reduced diversity departs from the healthy microbiome state [37, 43, 53, 55]. Notwithstanding this concept, the mere fact that bacterial microbiomes remain largely stable seems to contradict simplistic models of targeted antimicrobial elimination of pathogenic microbes as a basis for therapeutic efficacy. Furthermore, how antimicrobial agents such as macrolides, which have no activity against *P. aeruginosa*, offer the observed therapeutic benefit in bronchiectasis remains poorly understood. This may involve more indirect influences upon other microbial constituents and their associated community structure but extend to include the drug's anti-inflammatory and immunomodulatory effects [56].

Non-tuberculous Mycobacteria

The role of non-tuberculous mycobacteria (NTM) is important and warrants particular mention given its incidence as a cause and consequence in bronchiectasis including its associated and significant challenges in detection by NGS methodologies [16, 57, 58]. Bronchiectasis and NTM infection are highly correlated to airway distortion, which in itself is thought to predispose to NTM colonization and disease progression in bronchiectasis [59]. *Mycobacterium avium* complex (MAC) is the most prevalent NTM detected although geographic variation exists [8]. Analysis of US registry data reveals a significant burden of MAC, followed by *M. abscessus* and *M. chelonae* in bronchiectasis who develop symptoms in later life and who are predominantly female [16]. As such, the presence of NTM potentially represents a

sub-phenotype of bronchiectasis and a possible means of stratification towards microbiome-directed therapy. Importantly, however, recent work highlights the difficulty in a reliable detection of mycobacteria using targeted 16S rRNA sequence analysis whereby *Mycobacterium* spp. are frequently absent from 16S rRNA gene profiles in samples positive for mycobacterial cultures [57]. This discrepancy between culture-based and culture-independent analysis suggests a lack of sensitivity in culture-independent methods for the detection of mycobacteria. This likely reflects the relatively low copy number of 16S rRNA genes per genome that leads to an underrepresentation of these bacteria by targeted 16S analysis. Notwithstanding this technical limitation, Sulaiman et al. demonstrate the existence of distinct host phenotypes in NTM-positive bronchiectasis patients, where impaired IFN-γ and GM-CSF production is coupled to significant association with upper airway taxa and T-helper-17 cytokines [57]. These observations add credence to the concept of NTM infection as a potential endophenotype and treatable trait in bronchiectasis and is important given the association of NTM with susceptibility to fungal infection which leads to a complex microbiological picture in the airway and its associated treatment challenges [15, 39].

The Mycobiome in Bronchiectasis

Given the structural distortions of the airway observed in bronchiectasis, the risk of fungal exposure and subsequent colonization is increased. This results in an increased sensitization to fungal allergens and the occurrence of allergic bronchopulmonary aspergillosis (ABPA) [17]. As seen in other chronic respiratory disease states, fungal sensitization and allergy are associated with negative outcomes in bronchiectasis including poorer lung function and increased exacerbation [17, 60–62]. The immunology of fungal sensitization and its associated host response is extensively characterized in CF where *Aspergillus fumigatus* represents a key predominant airway fungal pathogen identified in association with Th2-driven response and antecedent to the emergence of ABPA that is accompanied by significant clinical symptoms and lung function decline [62]. While the host response to fungi in bronchiectasis remains lesser studied, marked increases in sensitization to *Aspergillus* antigens are noted as is the increased activity of the anti-fungal chitinase enzyme chitotriosidase (CHIT-1), both of which exhibit geographic variation [19, 63, 64]. While important, the diagnosis of fungal infection remains challenging due to the poor sensitivity and specificity associated with existing diagnostics and a lack of standardization between centres. This leads to delays in therapy and therefore adverse outcomes [62, 65]. The application of ITS amplicon sequencing has therefore been proposed and examined as an alternative detection method highlighting the complex fungal profiles seen in CF that appear distinct from non-CF bronchiectasis. In a head-to-head comparison, lower fungal diversity is observed in CF compared to non-CF bronchiectasis [18]. As fungal colonization, sensitization and ABPA all individually represent potentially ‘treatable traits’ in bronchiectasis, a

detailed characterization of the airway mycobiome is worthy despite the inherent technical challenges related to mycobiome analysis [39, 66, 67]. In the largest investigation of the bronchiectasis mycobiome performed to date, the Cohort of Asian and Matched European Bronchiectasis (CAMEB) compared mycobiome profiles generated by ITS amplicon sequencing in patients from Asian and European bronchiectasis cohorts 'matched' for age, sex and disease severity [19]. This important work provided the first broad insight into the airway mycobiome in bronchiectasis and identified host responses related to fungal presence that associate negatively with clinical outcomes. This study illustrated the higher abundance of potentially pathogenic taxa including *Aspergillus*, *Penicillium* and *Cryptococcus* in bronchiectasis (compared to healthy controls) and the presence of an unfavourable allergic sensitization and immune response profile associated with *Aspergillus*. The study design further allowed comparisons between the Asian (Singaporean and Malaysian) and European (Scottish) patients, matched by age, sex and Bronchiectasis Severity Index (BSI) score. This permitted a clear assessment of geographic differences in the mycobiome while controlling for disease severity. Differences in mycobiome profiles were identified including increased relative abundances of *Simplicillium, Trichosporon* and *Aspergillus* in the Asians, while a higher abundance of *Wickerhamomyces*, *Clavispora* and *Cryptococcus* distinguished Europeans. *Candida* was frequently observed across both cohorts at comparable frequency, while the Scottish cohort exhibited a higher prevalence of *Saccharomyces*, *Penicillium*, *Cryptococcus*, *Clavispora* and *Botrytis*. Further analysis using a validated qPCR method that included quantification of the various *Aspergillus* species present in the airway revealed a predominance of *A. terreus* in the Scottish patient group, while *A. fumigatus* conidial burden was greatest in Asians [19, 68]. While these findings cannot be generalized beyond the local regions studied, they do illustrate the geographic variation observed in bronchiectasis and highlight both the similarities and differences that can be uncovered when appropriately designed populations are compared [8, 19]. Further stratification of the CAMEB study participants by an immunological classification system that accounted for fungal presence and associated host biomarkers including *Aspergillus*-specific IgE and IgG and sputum galactomannan identified high frequencies of fungal sensitization and ABPA. These *Aspergillus*-associated disease states further revealed a clear association with disease severity, exacerbation frequency and lung function decline, particularly in those with serological ABPA (sABPA) [19]. This important, novel and clinically relevant observation was next further investigated using an extended panel of fungal allergens revealing a remarkable and very high level of sensitization among bronchiectasis patients [20]. Sensitization level and the occurrence of polysensitization was linked to poorer lung function but not exacerbations; however, assessment of the host airway immune response allowed a clustering of patients according to "immunoallertypes": one fungal driven and proinflammatory and a second characterized by sensitization to house dust mite coupled to chemokine dominance. Critically, the fungal patient cluster demonstrates greater disease severity and poorer lung function [20]. Current works characterizing the mycobiome and the sensitization response in bronchiectasis have thus far revealed that combining

immune profiling with patient clustering reveals novel disease endophenotypes that potentially may be amenable to tailored and personalized bronchiectasis therapy.

The Virome in Bronchiectasis

The virome represents the most challenging and therefore least well-described aspect of the human microbiome and has yet to be clearly defined in the lung [38]. In bronchiectasis, the role of viruses remains unclear; however, they have been detected in the airway, and emerging evidence supports a potential role in disease [21, 22, 69]. The virome may be considered from a number of perspectives in bronchiectasis: (1) the role of common respiratory viruses and their impact on health status, (2) acquired immunodeficiency associated with viral infection and (3) bacteriophages and their influence on the bacterial hosts, including as potential facilitators of horizontal gene transfer. The first two areas have been addressed in several existing studies, while the third represents a novel area in bronchiectasis yet to be meaningfully addressed by research. Considering what is known in other chronic respiratory disease states, viruses are considered important triggers of exacerbation. Gao et al. assessed the presence of viruses at exacerbation in a cohort of bronchiectasis patients from Guangzhou, China, representing the first large-scale prospective study determining the incidence and clinical impact of viral infection. Common viruses documented included coronavirus, rhinovirus and influenza A and B viruses. While systemic and lower airway symptoms were not significantly different between virus-positive and virus-negative exacerbations, several systemic and airway inflammatory markers (serum IL-6 and TNF-α; sputum IL-1β and TNF-α) distinguished virus-positive patients [22]. In subsequent work from Australia, a high frequency of stable bronchiectasis patients interestingly had viruses detected in their airways, particularly during winter (92%) compared to lower rates (33%) in the summer. The main viruses detected included rhinovirus, influenza A and B and respiratory syncytial virus with greatest incidence of co-infection in the winter months [21]. Both studies clearly confirm the presence of common respiratory viruses in the stable and exacerbation states in bronchiectasis albeit without a significant association to clinical outcome. Building on their previous work, Chen et al. next identified a significantly higher frequency of viruses at exacerbation compared to the stable state in patients from the Guangzhou region further implicating viruses in bronchiectasis exacerbations where rhinovirus and influenza A and B demonstrated the strongest effects [69]. The second aspect of virology that warrants consideration in bronchiectasis is the role of viruses as mediators of acquired immune deficiency, which in turn may accelerate the disease ‘cycle’ through disrupting normal immune homeostasis. Such viruses include the Human T-cell leukaemia virus, type 1 (HTLV-1) which has been documented in association with bronchiectasis in several studies, from western and indigenous Australian populations, the latter originally describing the link [23]. More recent evidence also implicates the Epstein–Barr virus (EBV), which is associated with a shortened time to next exacerbation and more rapid

decline of lung function [70]. While this latter association is less well supported, the concept of acquired immune deficiency should at least be considered and is consistent with current disease paradigms (Fig. 7.1). More recent virome research, especially in bronchiectasis relates to the role of bacteriophages and their contribution to microbiome architecture and stability. While WGS metagenomics has been performed in bronchiectasis, no study to date has directly assessed the presence and abundance of bacteriophages (Fig. 7.2) [50, 52]. Whether a distinct disease- or patient-specific bacteriophage profile exists in bronchiectasis remains to be established, and investigations should focus on characterizing the disease or specific patient groups, defined by bacteriophage pattern that associate with clinical outcomes. Emerging data from the gut in studies unrelated to bronchiectasis does suggest an individual specificity of the virome ('phageome'), with high inter-individual differences [71]. It is probably too premature to suggest whether such variability could be correlated with clinical outcomes in bronchiectasis, but as bacteriophages possess the potential to dramatically reshape the microbiome and contribute to dysbiosis, there is certainly scope for broad assessment from diagnostic and therapeutic perspectives [72, 73]. Additionally, the relevance of bacteriophages in the context of emerging antimicrobial resistance and as facilitators of horizontal gene transfer is highly relevant in respiratory diseases including bronchiectasis and remains a key area for future investigation [74].

Microbial Networks and the 'Multi-Biome'

While the bacteriome, mycobiome and virome have to date been considered as separate and individual entities, integrating them into a holistic 'multi-biome' framework appears to be the next logical step for bronchiectasis and other chronic respiratory disease states. The concept and description of the host microbiome as an integrated microbial network is emerging and has been advanced as a potential model underpinning exacerbations in CF [25]. Networks and their associated microbial interactions may better account for observed clinical differences compared to taxonomic abundance alone and therefore represents a promising platform for development of respiratory disease models of infection and exacerbation [75]. The role of fungi and their own inter-kingdom communication with bacteria remain a recognized part of a holistic ecosystem where active research is ongoing, and with clear relevance to bronchiectasis, as both kingdoms independently have been shown to be highly relevant in disease [8, 19, 41, 76]. Going beyond this, the role of viruses should also be considered, as individual common respiratory viruses, those linked to acquired immunodeficiency but also bacteriophages that can have a major influence on microbiome architecture and the mobilization of antimicrobial resistance genes. While each individual microbiome is clearly relevant, they remain to be fully investigated in the context of an integrated and holistic inter-kingdom microbial consortia, a rich avenue for future microbiome research in bronchiectasis.

Clinical Applications

The increasingly recognized heterogeneity of clinical endophenotypes in bronchiectasis helps to account for the failure of most clinical trials in this disease [36]. Given the associations between the microbiome and disease outcomes, it seems plausible to consider an integration of microbiome data into clinical trial design. This may prove beneficial, allowing for adequate adjustment of confounding microbiome-associated variables through appropriate patient stratification (Table 7.1) [41]. In addition to targeting microbial endophenotypes, the microbiome also offers potential as a secondary outcome measure and possible prognostic marker in assessing treatment effects directed at it. Surveillance of potentially undesirable changes in the microbial community such as the emergence of potentially pathogenic taxa or antimicrobial resistance genes is also of value [50, 52]. Host genetics is an additional key factor that, at least partially, predicts microbiome composition and should be integrated into patient stratification modalities where available [77]. Expanding the assessed microbial kingdoms in bronchiectasis through multi-biome analysis has already uncovered new perspectives, for example, the identification of fungal sensitization, a feature that may be to

Table 7.1 Clinical applications for microbiome research in bronchiectasis

Biome	Study	Method	Clinical correlate	Application
Bacteriome	Rogers et al. [49]	Targeted 16S rRNA analysis	Exacerbation frequency	Tailoring clinical trials to target most responsive patient subgroups
	Taylor et al. [77]	Targeted host gene sequencing 16S rRNA analysis	Exacerbation frequency, lung function, time to next exacerbation	Defining impact of host genetics on microbiome in patient stratification modalities
	Taylor et al. [50]	WGS metagenomics	Antimicrobial resistance	Tailoring clinical trials to limit emergence of resistance
Mycobiome	Mac Aogáin et [19, 20]	Targeted ITS analysis	Exacerbation frequency, lung function, severity (BSI)	Targeting fungal sensitization/airway immune response as a treatable trait
	Tiew et al. [80]	WGS metagenomics	Exacerbation frequency (COPD)	Design of environmental interventions
Virome	Chen et al. [69]	Targeted qPCR respiratory virus panel	Odds ratio for exacerbation/time to next exacerbation	Capture of viral status at exacerbation as potential risk indicator
	Shkoporov et al. [93]	WGS metagenomics	Not assessed (gut)	Charting of the respiratory 'phageome' in bronchiectasis
Trans-kingdom	Soret et al. [94]	Targeted 16S rRNA and ITS analysis	FEV1 % predicted (cystic fibrosis)	Integrative microbiomics to improve stratification and modelling based on co-occurrence networks

endophenotype-targeting precision medicine approaches [64]. Furthermore, the early application of metagenomics in bronchiectasis has already demonstrated a clear potential in terms of charting the emergence of antimicrobial resistance and the environmental exposome. The role of the bronchiectasis virome remains to be established; however, emerging large-scale studies do suggest an involvement with exacerbation risk. While an untargeted appraisal of the virome or 'phageome' associated with the bronchiectasis microbiome remains to be examined, the clear individualized and stable phage profiles recently determined in gut microbiomes provide a clear framework for future lung investigations and in particular the examination of bacteriophages in bronchiectasis. From a diagnostic perspective, in-depth analysis of the microbiome (integrating trans-kingdom analysis with temporally and anatomically distinct samplings) may provide the ultimate stratification system by which specific bronchiectasis subtypes or overlap syndromes may be robustly defined and in turn provide a more focused management and precision medicine approach in this disease state (Table 7.1).

Future Directions

Against the backdrop of ageing global demographics and an increased awareness of the disease, there is a growing appreciation of the clinical burden of bronchiectasis [36]. This has led to renewed focus on the disease and increased research including the increasing number of microbiome studies described in this chapter (Fig. 7.2). Analysis of the microbiome has uncovered the complexity of the microbial consortia in bronchiectasis and its potential for patient stratification according to profile rather that the simple presence or absence of individual taxa [40]. A major challenge, however, in the translation of these findings will be the standardization of methodologies across studies, including the integration of data from distinct microbiome profiles: i.e. profiles obtained using different platforms, i.e. bacterial vs fungal vs viral, or profiles from distinct anatomical sites, i.e. gut vs lung in a given patient [41, 78]. Integrative approaches for patient stratification based on multiple 'omic' datasets have shown early promise, and the further development and application of these approaches to 'multi-biome' datasets may provide even deeper resolution of patient microbiome subtypes in bronchiectasis [79]. Larger studies with greater numbers of patients (approaching those already achieved in culture-based studies – Fig. 7.2) will be required, and the inclusion of longitudinal sampling and comparing geographically distinct cohorts will help further in attaining the necessary resolution required for clinical use. We must ensure to continue to improve the methodologies used for microbiome analysis, making them more accessible, refined and scalable. While targeted amplicon sequencing has now been applied in several studies, metagenomic analyses of bronchiectasis remain limited, and metatranscriptomic analyses are yet to be described. The insights that may be derived from a functional appraisal of the metagenome remains an important area for future

work. Likewise, the virome and 'phageome' remain to be fully characterized in bronchiectasis. Recent work from our group demonstrates the potential of metagenomics to allow sampling of the environment (patient inhaler devices and both indoor and outdoor air) in addition to the airway microbiome for identifying important exposures such as resistome-harbouring microbes and airborne fungi to which exposed patients can demonstrate sensitization responses that associate with negative clinical outcomes [52, 80]. The air microbiome and that of the built environment are therefore emerging as important factors in respiratory health and are likely to be relevant to bronchiectasis [80–82]. Air pollution is a well-established risk associated with adverse outcomes in chronic respiratory disease including bronchiectasis including exacerbations and hospital admission [83–86]. In this context, the microbial composition of both indoor and outdoor air appear an important consideration, yet air remains an under-sampled and under-studied planetary ecosystem with potential relevance to respiratory disease [82, 83]. Metagenomics has yielded our first insight into the dynamic nature of the air microbiome revealing its composition and diel fluctuation in microbial content [82]. Furthermore, microbes have the propensity to persist in air, on surfaces and within water systems supplying the built environment, and the indoor microbiome is undoubtedly influenced by factors inherent to building design including the use of modern materials [81, 87]. It therefore seems logical that this could impact lung health as demonstrated in our recent work focused on fungal sensitization in COPD [80]. As sensitization also represents an important clinical correlate in bronchiectasis, comparable host-environment interplay is likely involved and amenable to metagenomic study [64]. This early work highlights great potential for environmental-related intervention studies in chronic respiratory disease states including bronchiectasis where modifying environmental factors could potentially provide a cost-effective and non-invasive alternative to pharmacological therapy. The application of such an approach to much larger bronchiectasis patient populations is desirable, where host and environmental metagenomes are characterized together, coupled to an assessment of the host response to better evaluate bronchiectasis endophenotypes in relation to their surrounding environment (Table 7.1).

While it is natural to focus on appraisal of the lung microbiome in chronic respiratory diseases, microbiome composition at other anatomical sites is also an important consideration. The oral microbiome – which forms a continuum with the upper and lower respiratory tract along an ecological gradient – is of relevance, as its composition may influence or predict immunological status in the lower airway or even the presence of other respiratory conditions [56, 88]. The composition of the gastrointestinal microbiome is also important both because of the potential for subclinical micro-aspiration of gut microbes and their accompanying inflammatory consequences. In addition, the interplay between the gut microbiome and immune homeostasis may further influence the pathogenic response to microbial encounters in the lung through the lung-gut axis [38, 89]. The role of gastrointestinal disorders such as gastroesophageal reflux disease (GERD) and irritable bowel syndrome (IBS), both reported as bronchiectasis comorbidities, may signal the presence of a dysbiotic gut microbiome [90, 91]. The development of integrative methodologies

for the sequential analysis of multiple biomes are now advancing, paving the way for analysis of multiple microbiome samples from individual patients that will likely provide even more granularity for patient stratification across patient cohorts and even anatomical sites [75, 92]. The era of microbiome medicine is arriving and learning from the lessons of the past; bronchiectasis must not be left behind.

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Chapter 8
COPD

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Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive inflammatory disease that irreversibly damages the lungs and can severely diminish a patient's quality of life. Further, COPD is a heterogeneous disease with two major phenotypes: one characterized by parenchymal destruction and loss of elastic recoil (emphysema) and a second characterized by goblet cell hyperplasia, increased mucus production, and bronchiolitis with lymphocytic and neutrophilic infiltration (chronic bronchitis). However, most COPD patients do not easily fit into this strict dichotomy but rather one that manifests as a mix of these phenotypes.

Multiple environmental factors contribute to the pathogenesis and natural history of COPD including environmental exposures, infections, inflammation, and genetic predisposition [1–3]. While tobacco smoking is the most common environmental risk factor in the USA, outdoor air pollution and poor indoor air quality from burning biomass fuels are major contributing risk factors worldwide [2]. Recent data suggest that the microbial environment existing within human mucosae is another environmental factor that can contribute to the pathogenesis of this disease. Using

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culture-dependent methods, airway colonization with bacteria has been associated with increased airway inflammation and accelerated airway obstruction among subjects with COPD [3, 4]. Further, COPD is characterized by altered host immune features that impair subject's ability to respond to microbes. For example, IgA deficiency is associated with COPD and can cause recurrent lower respiratory tract infections [5]; immunosuppression due to HIV is an independent risk factor for COPD [6, 7]; and the expression of Toll-like receptors 2 and 4 is altered in this disease [8, 9]. Additionally, as COPD progresses, lower airway colonization with potentially pathogenic microorganisms (**PPMs**), such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, or *Moraxella catarrhalis*, leads to further worsening of inflammation and disease progression [10–12]. Therefore, careful identification of biomarkers and phenotypes within COPD holds greater promise toward a more personalized therapeutic approach, and examining the respiratory microbiome may be one possible avenue. Hence, it is not surprising that with the use of culture-independent techniques over the last decade, we have begun to appreciate a much broader role of the microbiome in COPD.

Bacteriome in Stable COPD

The use of culture-independent approaches to study the microbes present in an environment (microbiota) has revealed that the lung mucosae, contrary to the classical view that it is sterile, harbors microbial products that are evidence for frequent exposure to multiple types of microbes. This should not have been surprising as silent aspiration is a common event in health, and its prevalence is increased in pulmonary disease such as COPD. In fact, laryngeal penetration and aspiration frequently occurs in individuals with stable COPD [13, 14]. Microaspiration is thought to occur due to an incoordination between breathing and swallowing [15]. Further, impaired mucosal immune response and frequent exacerbations also contribute to the bacterial colonization of the lower airways seen in patients with COPD as their disease progresses [16, 17]. Regular exposure to tobacco itself alters the microbiome of the oropharynx [18]. Interestingly, this dysbiosis in the oropharnyx does not automatically translate to dysbiosis in the lower airways, unless there is pre-existing impaired lung function, such as with COPD and asthma [19].

However, even in stable COPD, lower airway microbial colonization is associated with increased lower airway inflammation. Quantitative bacterial cultures of lower airway samples from patients with COPD have shown that lower airway colonization is associated with higher levels of neutrophils and inflammatory cytokines, such as IL-8, in the lung [20]. The prevalence of positive cultures in stable COPD ranges from 22% to 83% [10, 21, 22]. Using this approach, prior investigations have shown that as the bacterial burden increases in the lower airways of patients with COPD, there is acceleration of the forced expiratory volume in 1 second (FEV1) decline, increased comorbid conditions, more frequent symptoms, and

exacerbations [23–26]. In these prior investigations, using culture-dependant methods, non-potential pathogenic microorganisms (non-PPM) such as *Corynebacterium* spp., *Neisseria* spp., *Enterococcus* spp., coagulase-negative *Staphylococci*, *Streptococcus viridans*, and *Candida*, common commensals of the oral cavity are frequently found in the lung [10]. Among the potentially pathogenic microorganisms (PPM), *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* are the most frequent organism cultured [10, 12].

Even before the broad use of culture-independent techniques based on identification of conserved regions within broadly distributed bacterial genes, such as 16S rRNA gene sequencing, investigators noted that microbial products from PPMs were frequently found among COPD subjects whose respiratory secretions did not yield positive culture results [27–29]. In the last 10 years, with the use of high-throughput sequencing of the microbial 16S rRNA gene, we have a much more comprehensive view of the presence and relative abundance of both uncultivable and cultivable bacteria [30, 31]. Using this technique we now know that in moderate to advanced stage COPD, there is enrichment with Gammaproteobacteria (which includes many PPMs such as *Haemophilus* and *Moraxella,* Figure 1) and with oral commensals [30–32]. In a large study comparing 124 stable COPD subjects to 124 healthy subjects, *Haemophilus* was the most dominant genus in stable COPD, while *Streptococcus* was the most dominant genus in healthy subjects [33]. The authors also used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis to predict functional gene content based on 16S rRNA gene content. Interestingly, subjects with COPD had higher proportions of genes associated with bacterial motility proteins, lipopolysaccharide biosynthesis, ABC transporters, and secretion systems.

Furthermore, in a study of 253 clinically stable COPD patients, those with Proteobacteria-dominated microbes in their sputum microbiome had poorer lung function, worse symptoms scores (modified Medical Research Council dyspnoea score), and more frequent exacerbations and were more likely to be COPD GOLD Group D [34]. Importantly, the authors found that those subjects with *Haemophilus*-dominated sputum microbiome were associated with significantly higher mortality (hazard ratio [HR] 5 2.53; 95% CI 5 1.08–5.94; $P = 0.032$). The authors went further and evaluated sputum protein profiles with nanoflow liquid chromatography with tandem mass spectrometry. Among subjects with Proteobacteria-dominated sputum microbiome, there was a positive correlation with multiple proteins that are markers of neutrophilic inflammation, including myeloperoxidase catalase, matrix metalloproteinase 9, matrix metalloproteinase 8, and neutrophil elastase. Furthermore, by pathway analysis, the authors found upregulation of proteins along the neutrophilic activation pathway in those subjects with Proteobacteria-dominated sputum [34].

The microbiome in COPD also changes with disease severity. Comparing the sputum microbiome of 9 mild/moderate subjects to 10 severe COPD subjects; increased severity is associated with reduced microbial diversity [35]. Similar results were found in a similar study comparing 8 moderate to 9 severe subjects [36].

Virome in Stable COPD

While most investigations have focused on the bacterial component of the microbiome, respiratory viruses can also be found in stable COPD patients. For example, respiratory syncytial virus (RSV) is found in up to 23.5% of COPD patients, while a smaller fraction of patients will have rhinovirus, coronavirus, and parainfluenza virus detected by PCR in sputum [37]. Next generation sequencing has more recently been used to identify respiratory viruses [38]. It has been shown to be just as sensitive as real-time PCR and provides almost immediate information on virus typing [38]. Smoking itself has been shown to alter the lower respiratory virome. In a study comparing 20 current/former smokers to 10 nonsmokers, *Prevotella* phages were twofold higher in smokers, while *Lactobacillus* and *Gardnerella* phages were reduced in abundance among smokers [39]. Additionally, *Actinomyces, Capnocytophaga, Haemophilus Rhodoferax* phages, and eukaryotic DNA viruses were found only in smokers. Importantly, the authors were able to show a significant association of the respiratory virome in smokers with arachidonic acid and IL-8 levels, known factors in the pathogenesis of COPD [39].

Respiratory viruses also have a significant impact on the bacterial component of the respiratory microbiota. In one of the few experimental investigations made in a human cohort, investigators infected healthy volunteers and subjects with COPD with rhinovirus [40]. Evaluation of the sputum microbiota before and after rhinovirus infection showed that there was an increase in the overall bacterial load particularly with *Haemophilus influenzae* enrichment. This data supports the hypothesis that the respiratory virome is an integral part of the selection pressure existing on the respiratory microbiota and can foment the outgrowth of a respiratory pathogen.

Lower Airway Microbiota and the Inflammatory Process in Stable COPD

Chronic lower airway inflammation leads to parenchymal damage and airway obstruction. However, different immunological phenotypic patterns can be identified. Compared with smokers without COPD, those with COPD have higher levels of neutrophils, macrophages, B lymphocytes, and $CD4^{+}/CD8^{+}$ T cells in biopsies of peripheral airways, highlighting the role of innate and adaptive immune responses in disease progression [41]. While neutrophilic inflammation is classically described in COPD, between 15% and 40% of patients with this disease will exhibit an eosinophilic phenotype [42]. These patients may present with different clinical outcome and response to treatment. Identifying the specific type of inflammation/phenotype has become increasingly important in how physicians personalize treatment for this heterogenous disease. For example, there is now mounting evidence that in a subgroup of COPD, patients with predominantly neutrophilic inflammation, use of inhaled corticosteroids can lead to an increased incidence of pneumonia [43].

The role of smoking on the inflammatory process has been extensively studied, including preclinical models that provide us with mechanistic insights. Using animal models of COPD, investigators have been able to demonstrate that chronic exposure to cigarette smoke is associated with high numbers of cells producing IL-17A and IFN-γ in bronchoalveolar lavage (BAL) fluid, and IL-17A-dependent gene upregulation including IL-8, MMP12, and MCP-1 in lung homogenates [44, 45]. Furthermore, mice deficient for the IL-17 receptor are protected from cigarette smoke-induced emphysema [46]. However, the role of the lung microbiota on the inflammatory process is less well known.

In healthy subjects, the enrichment of the lower airway microbiota with oral commensals is associated with a Th17 inflammatory phenotype [47]. Interestingly, the alveolar macrophages of those subjects with increased relative abundance of oral commensals and increased inflammation display a blunted innate immune response to lipopolysaccharide (LPS). These data suggest that aspiration of oral secretions (containing the oral commensals) leads to increased inflammation and concomitant counter regulatory immune mechanisms. These changes may affect the immunological pressure on the lower airway microbiota (and the PPMs on it). Increased lower airway inflammation and mucus production can lead to increased nutrient supply to bacteria in the lung [48]. In conjunction, blunting of the inflammatory response to LPS may make the response to gram-negative bacteria less efficient.

Others have evaluated the lower airway microbiota and host immune phenotype in advanced stage COPD. Using lung explants from patients with GOLD Stage 4 COPD and non-COPD donors, investigators have found that low alpha diversity is associated with increased emphysematous destruction, remodeling, and increased $CD4^+$ T cells [49]. *Haemophilus influenzae* (Proteobacterium) was the most differentially enriched when comparing non-COPD to stage 4 COPD donors. Importantly, differences in the relative abundance of Firmicutes and Proteobacteria (phyla containing several respiratory PPMs) were associated with different transcriptomic signatures. Neutrophil infiltration was negatively associated with the presence of Proteobacteria, Comamonadaceae, *Pseudomonas*, and Betaproteobacteria, while eosinophilic infiltration was positively associated with Actinobacteria. In addition, sputum samples from 43 COPD and 16 control subjects identified *Haemophilus* and *Moraxella* as strongly associated with host immune profile [50]. Interestingly, *Haemophilus* was associated with both stable state and exacerbating COPD; *Moraxella* was exclusively associated with COPD exacerbations.

Human studies are commonly limited by its cross-sectional design and/or difficulties in conducting controlled experimental interventions, making it hard to establish causal inferences. Therefore, the use of preclinical models may offer some support to causality. Animal models of COPD have shown that the lower airway microbiota can play a significant role on the modulation of the lower airway inflammatory tone and airway/parenchymal damage. For example, using an LPS/elastase preclinical models of COPD, investigators have shown that lower airway dysbiosis can be partially responsible for the Th17 lower airway inflammatory tone [51]. Secretory IgA deficiency has been shown to alter the lower airway microbiome and

the development of COPD-like pathological deragements [52]. Interestingly, in a longitudinal investigation using macaques with Simian-Human Immunodeficiency Virus (SHIV), an infection that is associated with COPD in these animals, development of obstructive lung disease was characterized by a relative increase in abundance of oral anaerobes such as *Fusobacterium*, *Prevotella*, *Veillonella*, *Neisseria*, and *Porphyromonas* [53].

Resistome in Stable COPD

Part of the management of COPD involves the use of anti-microbial medication, particularly for infective exacerbations. However, since the advent of antibiotics, resistance to these medications has become a global public health issue, the era of the reisistome [54]. Metagenomic assessment can also be used to profile the presence of specific antimicrobial functions (so-called resistome). Few studies have examined the resistome in chronic lung disease. In a study that compared healthy subjects ($n = 13$) to subjects with asthma (=11) and subjects with COPD ($n = 15$), those with COPD had the highest abundance of multi-drug resistance genes, encoding multipartite efflux systems and regulatory proteins [55]. Interestingly, *ermX*, a macrolide resistance gene, was significantly increased in those with chroinc lung disease. In a larger study, using sputum samples obtained from 85 subjects with COPD (as well as some with asthma, bronchiectasis, and controls) investigators performed metagenomic analysis. Functional classification identified an abundance of carbohydrate and amino acid–related pathways toward lipid associated pathways in subjects with COPD and bronchiectasis. The authors also found alteration in genes associated with antibiotic-associated pathways including degradation, detoxification, and antimicrobial resistance. Even though subjects with COPD had the highest diversity of resistance genes, the investigators found that antibiotic resistance genes are commonly found even among those never exposed to antibiotics [56]. Among those genes, the highest prevalence of antibiotic resistant genes were for macrolides, followed by β-lactams, fluoroquinolones, and tetracyclines. Furthermore, a study comparing 55 COPD to 29 controls found a higher prevalance of antimicrobial resistance genes in COPD subjects [57]. Interestingly, the prevalence of these genes correlated with total bacterial abundance and not with lung function. As discussed earlier, antibiotic therapy is commonly used in chronic lung diseases such as COPD, asthma, and bronchiectasis. And in some instances, chronic macrolide therapy, for both its anti-microbial and anti-inflammatory properties, has been shown to be effective in reducing exacerbations [58]. Understanding the respiratory microbiome and resistome in these groups of patients will be pivotol in understanding changes in antimicrobial resistance over time [59].

One class of antibiotic commonly used in COPD is macrolide antibiotics, such as daily azithromycin to reduce exacerbation frequency. The effects of such chronic therapy on the lower airway microbiome and resistome is not well known. In a pilot placebo-controlled clinical trial, the use of azithromycin was evaluated in subjects

with early emphysema [60]. The lower airway microbiota and cytokine levels were evaluated using bronchoscopic samples obtained before treatment with azithromycin (250 mg daily) or placebo and was repeated after 8 weeks. Investigators found that azithromycin was associated with a reduction in α diversity (but not the bacterial load) and compositional changes in the lung microbiota that contrasted with the relative stability found in the placebo group. In addition, azithromycin treatment led to a decrease in inflammatory markers in the lower airways. Concomitant evaluation of the lower airway metabolome identified several microbial metabolites with immunodulatory properties. Evaluation of the effects of these bacterial metabolites in alveolar macrophages in ex vivo documented the anti-inflammatory effects affecting the same inflammatory markers identified in the human cohort. In contrast, ex vivo exposure of alveolar macrophages to azithromycin did not lead to significant changes in the same inflammatory markers. This data supports the theory that part of the anti-inflammatory effects observed with azithromycin may be mediated by its direct antibiotic effect – affecting microbial metabolism and leading to the release of bacterial metabolites with direct anti-inflammatory effects. More detailed investigations into how chronic antibiotic therapy in this population of patients affects the resistome is required.

Effects of Inhaled Medications on the Lower Airway Microbiota

Another important treatment modality in chronic lung disease is inhaled medications such as inhaled corticosteroids (ICS) and inhaled beta-agonists. Inhaled corticosteroids are associated with compositional changes in the lung microbiota [32]. Its use in COPD, while aiming to reduce airway inflammation, has constantly been questioned. This is partly due to the lack of effectiveness associated with definitive clinical outcomes (such as mortality), especially in milder disease, but also due to concerns for increased risk of pulmonary infections [61]. In subjects with moderate to severe COPD, triple therapy with inhaled corticosteroid, long acting beta-agonist (LABA), and long acting antimuscarinic agent (LAMA) was recently associated with a reduction in acute exacerbations of COPD (AECOPD) compared with LABA/LAMA combination without inhaled corticosteroid [62]. However, the incidence of pneumonia has been shown to increase with the use of inhaled glucocorticoids. In a study of 23 subjects with stable COPD, ICS lead to a significant increase in the relative abundance of *Streptococcus* when compared to non-ICS users [63]. The authors of this paper were able to also show that ICS impairs clearance of *Streptococcus pneumoniae,* a pathogen known to cause COPD exacerbations. This impaired clearance appeared to be due to supression of an important anti-microbial peptide, cathelicidin. Nonethless, further investigations are needed to evaluate the change in the lower airway microbiota after initiation of inhaled medications.

Acute Exacerbations of COPD (AECOPD)

AECOPD, defined by a sustained increase in respiratory symptoms that frequently requires treatment with steroids and antibiotics, leads to a significantly increased rate of morbidity and mortality. Patients that have two or more exacerbations per year have more rapid lung function decline and worse outcomes [64, 65]. The role of respiratory pathogens in the pathogenesis of AECOPD has long been documented with culture-dependent methods, and culture-independent methods are now providing a new dimension. Respiratory bacterial pathogens are recovered under aerobic conditions in about half of patients with AECOPD; respiratory viruses are isolated on 30% and co-infection with viruses and bacteria occurs 25% of the time [12, 66]. The most common bacterial pathogens found in AECOPD are *Haemophilus influenzae, Streptococcus pneumoniae*, and *Moraxella catarrhalis*. However, with culture-independent techniques, molecular profiling of sputum isolates has shown that acquisition of new bacterial strains frequently precedes AECOPD [67]. In a longitudinal study of 281 subjects with COPD [68], the authors found that dysbiosis in the sputum microbiome was associated with an increased severity in an exacerbation (greater fall in the forced vital capacity) as well as a significant increased symptom score (COPD assessment test). Interestingly, in comparing asthma exacerbations to COPD exacerbations with next generation sequencing, varying exacerbation phenotypes are identified, some with overlap between the two obstructive airways diseases [69]. In a COPD predominant cluster, patients had increased sputum neutrophil counts, IL-1b, IL-6, IL-6 receptor, TNF-a, TNF receptors 1 and 2, and vascular endothelial growth factor in association with an increased abundance of Proteobacteria.

Current treatment for AECOPD includes steroids alone or steroids with antibiotics for infective exacerbations, both of which have an impact on the lung microbiome. In a study of 8 subjects admitted to an intensive care with an AECOPD [70], treatment with only oral steroids resulted in an increased bacterial burden, with enrichment of Proteobacteria, Bacteroidetes, and Firmicutes. Not surprisingly, treatment with antibiotics alone reduced bacterial abundance, particularly for Proteobacteria.

There are several challenges with studying patients' lung microbiome changes in AECOPD. Firstly, patients frequently receive early treatment with systemic steroids and antibiotics that skew the evaluation of the airway microbiota. Further, sampling is frequently restricted to sputum samples since it is often difficult to perform lower airway sampling with bronchoscopy when patients are not in a stable respiratory condition. When the airway microbiota is evaluated during AECOPD using sputum samples, AECOPD is associated with decreased microbial diversity and increased proportion of Proteobacteria [71, 72]. Random forest modeling has also been used to differentiate AECOPD from stable COPD [73]. In this modeling, bacteria commonly found in the upper respiratory tract such as *Gemella, Porphyromonas, Haemophilus, Neisseria*, *and Streptococcus* were enriched in stable COPD when compared to AECOPD. Further, *Pseudomonas* was more abundant in AECOPD as

compared with stable COPD. Moreover, Wang et al. showed that changes in lung microbiota are associated with positive bacterial cultures, elevated eosinophils, and levels of IL-8 in sputum, a key neutrophil chemo attractive cytokine [71]. However, it is interesting to note that not all exacerbations have the same inflammatory phenotype, and while neutrophilic inflammation is classically described in AECOPD, a subset of patients will have exacerbations characterized by airway eosinophilia [74]. Interestingly, again in the paper by Wang et al. [71], there were no consistent differences between the sputum microbiota in stable disease vs. the sputum microbiota during AECOPD. However, within samples obtained during AECOPD, enrichment of the sputum microbiome with Proteobacteria was associated with culture positivity while enrichment with Firmicutes was noted among those subjects with elevated sputum eosinophils. Similar results were found in a subsequent paper [75] and, considering that different COPD phenotypes likely have significant clinical implications, it would be interesting to test the utility of the airway microbiota as a potential biomarker. Indeed, some investigations have proposed that the sputum microbiota at the time of hospital admission for AECOPD seems to be associated with 1-year mortality follow-up [76]. The sputum microbiota of survivors was associated with enrichment with *Rothia*, *Prevotella*, *Veillonella*, *Fusobacterium*, and *Actinomyces*, while in non-survivors, the sputum microbiota was enriched with *Staphylococcus* and *Escherichia-Shigella*. Further studies are needed to address the validity and utility of these measurements as possible biomarkers. However, a major difficult of studying the microbiota during these exacerbations is the multiplicity of possible confounders occurring. For example, systemic steroids used during AECOPD are associated with enrichment of the sputum microbiota with *Haemophilus* and *Moraxella* [71], while antibiotic treatment is associated with decreased relative abundance in Proteobacteria [72]. The combination of antibiotic and steroids lead to an increase in the abundance of Proteobacteria and an increase in microbial diversity.

Should the role of bacteria in AECOPD be redefined? In a classical view, bacteria play a role through acquisition of a new pathogen and active infection or through chronic colonization with a pathogen. We now know that even if a pathogen is identified during AECOPD (as a new infection or chronic colonization), they are not in isolation. Further, as discussed above, multiple bacteria can be identified in airway samples where cultures are negative. Another method of antimicrobial defense is the formation of neutrophil extracellular traps (NETs) [77]. Recent studies have found NETs in the sputum of both stable and exacerbating COPD [78]. Further studies have shown increasing levels of NETs associated with disease severity [79]. In a recent paper by Chalmers et al., the authors were able to show that higher levels of NETs in sputum were associated with lower microbial diversity [80]. Interestingly, in a subset of 24 subjects with AECOPD, sputum samples prior to treatment with increased abundance of *Haemophilus* had significantly higher levels of NETs. With these findings, the authors speculate the use of NETs as a marker of airways dysbiosis in COPD.

What about viruses? It is known that viruses affect bronchial epithelial cells, airway smooth muscle cells, fibroblasts, and alveolar macrophages leading to

activation of transcription factors and several pro-inflammatory cytokines and chemokines [81]. The effects of the virome during AECOPD are less well studied. The respiratory viruses most frequently described include rhinovirus (by far the most prevalent one), coronavirus, para-influenza, adenovirus, and influenza virus [37, 66, 82]. In a study of 63 patients with 88 exacerbations, 23 samples were positive for viruses by qPCR [83]. By metagenomic sequencing, 22/23 samples were also positive for viruses. Viruses identified included rhinovirus C, parainfluenza 3, and influenza A. With metagenomic sequencing, additional viruses identified included herpes simplex virus type I and coronavirus OC43. Metagenomic sequencing also offers additional viral typing that qPCR does not. For example, of the 13 rhinoviruses identified by metagenomic sequencing, the authors were able to type the virus to rhinovirus A (46.2%), rhinovirus B (15.4%), and rhinovirus C (38.5%).

The effects of the virome on the bacterial microbiota is also likely important. For example, for many years, clinicians have identified that viral infections can precede bacterial infections. Studies have shown that viruses can lead to higher bacterial burden, more sputum eosinophils, greater lung function impairment, and longer hospitalization [66, 84]. This synergism between viruses and bacterial pathogens can reflect their ability to trigger different host immune inflammatory pathways leading to severe presentations of AECOPD.

Research Needs in this Area

It was recently highlighted in a review article on COPD that although there have been a lot of advancements in the management of COPD, there remains poor understanding of phenotyping and endotyping of this disease [85]. Studies into the lung microbiome of these patients may help us in this process. However, many of the studies performed in COPD have focused on moderate to advanced stages of the disease and, therefore, might be confounded by the use of antibiotics and corticosteroids, frequent treatments for such patients (Fig. 8.1) [30–32, 70]. The changes in the lung microbiota that occur in early stages of the disease, or with COPD treatment and how those changes affect the disease process, are unclear. Further, the severe structural disease seen in some cases of COPD leads to the use of lung transplantation as a last resource. Longitudinal studies are needed to see how the change in lung microbiome over time affects prognosis and graft survival in this select cohort of patients. Although the study of the lung virome is in its infancy, more research in its role in stable COPD and COPD exacerbations is needed. Furthermore, the lung mycobiome, the study of fungi, is also a relatively new field, and very few studies have looked at its impact on COPD [86, 87]. Additionally, the interaction between smoke inhalation injury with the development of COPD and how alteration in the lung microbiome may impact on this disease has yet to be fully explored. Preclinical models focusing on modulation of the lower airway microbiota will be key to generate mechanistic insights into the role of the lung microbiome on the development of COPD. Going forward this may lead to novel therapies where

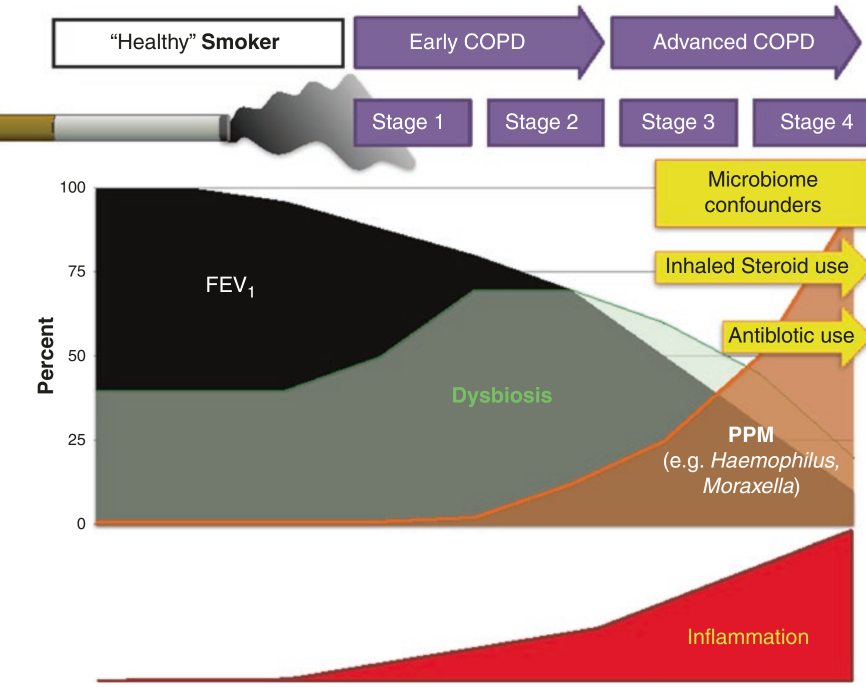

Fig. 8.1 Schematic of the role of the lung microbiome in COPD development

alteration of the lung microbiome may help prevent or even halt lung function decline seen in COPD. Putting all these approaches together, the comprehensive evaluation of microbial and host material is quickly transitioning from the research to clinical applications, the field of clinical metagenomics [88, 89]. It has the potential to change how physicians treat and manage patients with chronic diseases such as COPD. However, more research in COPD is required to reach a point of a more personalized approach based on the individual's lower airway microenvironment.

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Chapter 9
Idiopathic Pulmonary Fibrosis and Other Interstitial Lung Diseases

Rachele Invernizzi, Stavros Garantziotis, and Philip L. Molyneaux

Introduction

Interstitial lung disease encompasses a wide range of conditions characterised by a combination of interstitial inflammation and fibrosis [1]. These conditions are thought to arise in genetically susceptible individuals who encounter the right environmental exposure [2]. These include occupational exposure such as asbestos, hypersensitivity pneumonitis reactions secondary to mould or birds, gastro-oesophageal refluxate or underlying autoimmune diseases such as rheumatoid arthritis. However, the disease also manifests in the absence of any known triggers or cause and is then termed idiopathic.

While the phenotype of disease differs based on the exposure, the underlying genetic susceptibility and association with specific alleles remains similar across the common forms of pulmonary fibrosis [3, 4]. In this model, individuals with rare, highly penetrant mutations can develop the disease without the need for an environmental trigger, while those with the more common risk allele require these environmental stimuli to develop disease (Fig. 9.1) [5].

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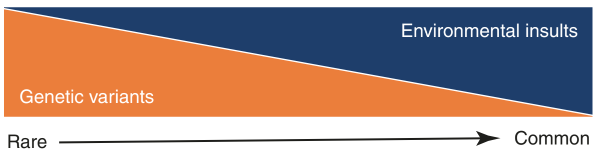

Fig. 9.1 The interplay between host and environment in interstitial lung disease. Those with rare, highly penetrant genetic variants need little input from the environment to develop disease, compared to those with more common abnormalities, where environmental triggers drive the disease phenotype. (Adapted from [5])

Even in the non-idiopathic conditions, the trigger for disease can remain elusive, such as in chronic hypersensitivity pneumonitis, where the offending antigen is not identified in almost half the cases [6]. Studies over the past decade have pointed to disordered host defence and thus susceptibility to infection, as an important contributor to disease progression in ILD [7]. Historically, viruses have been suspected of playing a role in the pathogenesis of pulmonary fibrosis, although the evidence supporting this, even during acute exacerbations, is scant and often conflicting [8]. The data implicating the role of bacteria in these processes was, until recently, also lacking. However, a number of well-designed prospective trials have allowed us to reconsider the potential role for the respiratory microbiome in fibrotic lung disease.

In this chapter, we will explore the role of the lung microbiome in pulmonary fibrosis, reviewing the evidence to date and highlighting areas of unmet need and future research priorities.

Idiopathic Pulmonary Fibrosis

It is now well recognised that resident microbial communities play a fundamental role in shaping healthy lung function and that disturbances in their composition are commonly observed in respiratory diseases including ILD [9–12]. The first study to investigate the presence of bacterial growth in the lower airways of subjects with IPF was conducted in 22 individuals and confirmed the presence of known pathogens including *Haemophilus*, *Pseudomonas*, and *Streptococcus* in 36% of the cases [13]. Over the past 5 years, advances in high-throughput molecular sequencing techniques have been used to characterise and interrogate the microbial composition in the lower airways of IPF patients as well as in animal models of the disease. These studies have consistently established the presence and importance of bacterial communities in the lungs of patients with IPF, suggesting that a perturbed microbial state may be linked to disease outcome and disproving the incorrectly held notion of sterility of the lungs outside of clinical infection. Bacterial communities, present outside the context of overt infection, have been hypothesised to act as a continued driver of epithelial injury. It is now recognised that an increased bacterial burden, loss of microbial diversity due to outgrowth of certain pathogenic

bacteria, is implicated with disease progression of IPF [9, 10]. Moreover, studies have also found that baseline bacterial burden can predict the rate of disease progression and risk of death [9]. Interestingly, several studies have suggested that the distal fibrotic lung is a microbial 'desert' [14, 15]. This contrast between abundant dysbiotic microbiome in the conducting airways and aerated lung units on one hand, and absence of microbes in fibrotic lung on the other, is an intriguing finding which may provide clues into the pathogenesis of IPF and the location of the early fibrotic foci.

The first study to characterise the lung microbiome in patients with IPF was the Correlating Outcomes with Biochemical Markers to Estimate Time-Progression (COMET) study [10]. The authors found a significant association between the abundance of specific microbial community members and disease progression even following adjustment for confounding factors. Specifically, an increased abundance of *Streptococcus* or *Staphylococcus* species was associated with a reduced progression-free survival time. However, the increased presence of these two bacteria was observed in less than half of the cohort, making it unlikely that these organisms alone can explain the disease pathogenesis. In a prospective study, Molyneaux and colleagues evaluated the microbial composition in BAL in patients with IPF, COPD and age-matched healthy controls [9]. Subjects with IPF were found to have an increased bacterial burden which was associated with disease progression (an association that has since been replicated) [14], as well as an increased expression of genes related to host defence. These patients also displayed significantly less diverse bacterial communities and were found to harbour a greater abundance of potentially pathogenic *Haemophilus*, *Neisseria* and *Streptococcus* species. In a separate cohort, the same authors found that the bacterial burden was also increased in AE-IPF subjects compared to patients with stable disease [16]. Despite showing no evidence of infection in clinical cultures, patients experiencing AE-IPF exhibited distinct changes in microbial community composition, such as an increased relative abundance of potentially pathogenic *Stenotrophomonas* and *Campylobacter* species, compared to stable disease. These organisms are potential respiratory pathogens, and the presence of *Campylobacter*, a bacterium usually confined to the gastrointestinal tract, led the authors to hypothesise that micro-aspirations and increased bacterial immigration to the lungs, may play a role in AE-IPF. Although this study offers an insight into the potential aetiology of AE-IPF, the patient cohort was relatively small, and there were only two paired samples from the same individuals when stable and experiencing an exacerbation. Therefore, future studies comparing samples from the same individual at baseline and during an acute exacerbation will be needed to allow for a more accurate insight into the biological changes underlying these events.

Collectively, these studies have established the presence of a lung microbiome in IPF and have highlighted specific alterations that occur compared to health. Nonetheless, these findings have not established the causal significance of the lung microbiome in IPF and fail to provide any insight into the functional mechanisms underlying the host-microbial interactions that may drive IPF pathogenesis and progression. Moreover, these studies fail to address whether the lung microbiota in IPF

are merely an indirect index of severity and therefore reflect an intrinsic, unmeasured feature of the disease.

Is the lung microbiome only an epiphenomenon in IPF, an innocent bystander to disease pathogenesis, or do altered respiratory microbiota directly participate in disease pathogenesis? This question was the rationale behind a recent study by Invernizzi and colleagues [11]. The authors quantified the severity and extent of fibrosis, including key features such as traction bronchiectasis and honeycombing. These radiographic findings were chosen given their known prognostic importance and because they provide objective insight into the anatomic distortion of IPF that may cause increased lung bacterial burden. Lung bacterial burden, while highly variable across patients, was unrelated to the radiographic severity of disease, whether considered collectively or in its specific features. Similarly, lung bacterial burden was uncorrelated with physiological severity of disease, as measured via forced vital capacity. In the same cohort, lung bacterial burden predicted clinical outcomes, defined as all-cause mortality or spirometric progression at 12 months even following multivariable adjustment for key potential confounders that represent radiographic and physiologic severity of disease. Collectively, these findings provide evidence that the lung microbiome's prognostic significance is not merely an artefact of disease severity [11]. A study by Huang and colleagues [17] demonstrated that a change in the activation status of innate immune pathways (NOD, Toll-like and RIG1) was associated with microbial abundance and diversity and was linked to fibroblast activation. Intriguingly, activation of the innate immune receptor TLR9 (which recognises unmethylated bacterial and viral DNA) in fibroblasts has been also linked to rapid IPF progression [18], lending further support to the notion that immunity-microbiome interactions may play a role in IPF development and progression in some patients.

In another study, O'Dwyer and colleagues [19] provided a link between microbial alterations in the lung, disturbed alveolar inflammation and IPF progression. Specifically, they found that alveolar inflammation was perturbed in patients with IPF and that a reduction in bacterial diversity promoted proinflammatory and profibrotic signalling profiles in the airways of these patients. Using a germ-free (GF) mouse model of fibrosis, the authors set out to explore the role of bacteria in alveolar inflammation and fibrinogenesis. Interestingly, they found that the absence of microbiota in GF mice was associated with altered humoral and cellular immunity and reduced mortality compared to conventional mice, despite exhibiting similar levels of fibrosis. This led the authors to suggest that IPF subjects not only die from progressive fibrosis but also from distinct inflammatory causes of acute-on-chronic respiratory failure, including respiratory infections and AE-IPF [19]. Overall, this study provided the first causal evidence that the microbiome is involved in the pathogenesis and mortality of fibrotic lung disease and that preclinical GF models are a useful tool to investigate potential mechanisms of host-microbiota interactions. Another recent study [20] used the bleomycin model to demonstrate that the lung, as opposed to gut microbiome dysbiosis, was responsible for the progression of fibrosis. These authors suggested that a specific immune pathway (MyD88-to IL-17B) was being activated by dysbiotic microbiome, thus leading to fibrosis via

immune activation. Although these results need to be replicated, and there will probably be more than one immune pathway contributing to fibrosis, these studies nevertheless suggest that microbial dysbiosis may promote adverse outcomes in lung fibrosis by inducing an adverse immune activation profile.

However, the concise mechanism of the respiratory microbiome in the pathogenesis of IPF remains unclear, and there is a pressing need for functional and longitudinal studies which will allow determination of more targeted treatments for lung diseases including IPF. Additionally, the fibrotic lung microbiome has not been fully characterised, and future studies should also characterise organisms other than bacteria and viruses, including fungi.

Hypersensitivity Pneumonitis

Hypersensitivity pneumonitis is an immune-mediated interstitial lung disease that develops in genetically susceptible individuals after repeated inhalation of organic antigens, including fungal, bacterial, animal and insect proteins [21]. HP can be classified into acute, subacute and chronic forms, depending on the inciting antigen, the intensity and duration of exposure and host factors [22]. Of these, it is chronic hypersensitivity pneumonitis (CHP) which carries the most significant morbidity and mortality. The chronic (or fibrotic) form of the disease arises following continuous exposure to an inhaled antigen, which initially elicits inflammation and ultimately evolves into irreversible and often progressive fibrotic lung disease [23]. There are no currently established international guidelines for the diagnosis of CHP, and it remains unclear why only a few exposed individuals develop the disease [24]. This diagnostic uncertainty is further confounded by the fact that, once advanced, the clinical features of CHP and IPF can be often indistinguishable [25]. Nonetheless, patients with CHP differ in prognosis, in the presence of an environmental antigen, and response to immunosuppression, suggesting distinct differences between these ILDs that remain to be elucidated.

Until recently no study had investigated the composition of microbial communities in the lower airways in CHP. This was the rationale behind a recently published study by Invernizzi and colleagues [12]. The authors compared the respiratory microbiome of healthy individuals with that of subjects with CHP and IPF. Here, the authors found that patients with CHP exhibited significantly lower lung bacterial burden compared to patients with IPF, although they still had greater lung bacterial burden compared with healthy subjects. Furthermore, the authors observed distinct differences in lung microbial composition between CHP and IPF. The lung microbiota of patients with IPF showed greater abundance of *Firmicutes* and lower abundance of *Proteobacteria* compared with CHP. At the genus level, *Staphylococcus*, was more abundant in subjects with CHP compared with IPF. However, unlike IPF, the abundance of *Staphylococcus* was not associated with clinical outcomes in the CHP cohort. Overall, this paper supports the hypothesis that IPF pathogenesis is uniquely impacted by the microbiome and that the increased bacterial burden

reported in IPF does not simply reflect the extent of underlying tissue fibrosis. However, this study has a number of limitations that must be acknowledged. The sample cohort is limited in numbers, and there are substantial differences in the patient cohorts, including significant differences in age, sex and disease severity at baseline. Furthermore, the study design does not take into account for the longitudinal changes occurring in the respiratory microbiome, and consequently key differences may be missed as a result. Nevertheless, this is the first study to explore the composition of the microbial communities in the lower airways in CHP and to show that, unlike in IPF, an increased bacterial burden in the respiratory tract of patients with CHP is not associated with mortality. A better understanding of this observation will necessitate further mechanistic and longitudinal work in order to further advance us toward more targeted treatments for these ILDs.

Other Interstitial Lung Diseases

Although the majority of our knowledge to date, regarding the impact of the lung microbiome in fibrotic lung disease, pertains specifically to IPF, a number of studies have begun to characterise the lung microbiome in other ILDs other than IPF. Sarcoidosis is an ILD of unknown aetiology that may be influenced by lung microbiota composition [26]. The microbiota of the lower airways of subjects with sarcoidosis has been found to be characterised mainly of *Prevotellaceae*, *Streptococcaceae* and *Acidaminococcaceae* [27]. Furthermore, an increased abundance of *Atopobium* and *Fusobacterium* has been found in sarcoidosis samples compared to healthy controls using 16S rRNA sequencing [28]. In a study by Clarke and colleagues [29], metagenomic sequencing revealed elevated levels of certain bacterial and fungal orders in single sarcoidosis sample types but did not detect enrichment of same orders across multiple sample types. In a separate study, the microbiome composition in bronchoalveolar lavage fluid of sarcoidosis patients was compared to that of individuals diagnosed with rheumatoid arthritis. The authors found similarities in the microbiota composition across the two cohorts and attributed the distal airway dysbiosis to a reduced presence of *Actinomyces* and *Burkholderia* [30].

Studies in subjects with systemic sclerosis, also known as scleroderma, have highlighted the importance of the gut microbiome. Scleroderma is an immune-mediated rheumatic diseases that is characterised by vasculopathy, fibrosis of the skin and internal organs, such as gastrointestinal tract and the lungs, and often progresses to ILD [31]. Dysbiotic intestinal microbiota has also been shown in patients with systemic sclerosis, in particular in those with extra-intestinal manifestations, including lung fibrosis and silicosis [32, 33]. Indeed, manipulation of the intestinal microbiota via early-life antibiotic administration was found to associate with dysregulated T-cell responses in the lung as well as altered expression of fibrosis-related genes [34].

Conclusions and Future Work

Although there are intriguing clues, from clinical and preclinical studies, of the potential role of the microbiome in the pathogenesis of interstitial lung disease, and more specifically IPF, there are still many gaps to be filled. The precise mechanism of microbial effects in IPF is all but unknown, and the clinical evidence, albeit compelling, is not proof of a mechanistic connection. We believe that the role of the microbiome in IPF pathogenesis must be understood in the framework we described in our introduction: 'genetically susceptible individuals who encounter the right environmental exposure.' Microbial dysbiosis is therefore an environmental exposure which will contribute to IPF in the right context and in susceptible individuals. Thus, the investigation of patient-microbiome interactions in IPF must encompass not only microbiome analysis but also investigation of the patient genetics and immune status. There is little doubt, in our mind, that IPF endotypes exist, and treatments will eventually be tailored to specific patient multiomic phenotyping [35]. Our conceptual approach may explain the conflicting results of antibiotic treatment in IPF patients [36–40]: treatment of "all-comers" should never be envisioned as a viable path in IPF, because it is highly unlikely that all patients will experience the host-microbiome interactions that lead to IPF. Conceptually, IPF patients with the right genetic background (e.g. polymorphisms in host defence genes, such as TOLLIP or MUC5B), evidence of dysbiosis *in addition to* evidence of immune dysregulation, would be the most likely candidates to benefit from an antibiotic intervention. Further translational studies into the mechanisms of microbiome effects in ILD will contribute to a precision medicine approach in this devastating disease group.

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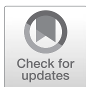
Chapter 10
Immune Suppression in Lung Disease: Lung Transplantation and HIV



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Introduction

Healthy respiratory function depends on the mutualism between the respiratory microbiota and human host through innate and adaptive immunity. Converging evidence from observational studies in humans and mechanistic studies in preclinical models suggests that this balance is disturbed in lung transplantation and HIV infection. In transplantation, dysbiosis is driven by the advanced lung disease

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necessitating transplantation, by the post-transplantation immunosuppression and antimicrobial exposure, and the development of alloimmune responses. In persons living with HIV (PLWH), advanced disease leads to a dysbiotic microbiome in the gastrointestinal (GI) tract and lung and increased susceptibility to both common and opportunistic infections. Even with effective antiretroviral therapy (ART) and preserved CD4+ counts, individuals have increased susceptibility to chronic obstructive pulmonary disease (COPD) which may be influenced by the microbiome.

The Lung Microbiome in Lung Transplantation and HIV

Studies of the healthy lung microbiome propose a separation into “pneumotypes” based on whether the predominant taxa are similar to those found in the oral cavity or not [1–5]. The latter has typically been considered to represent background flora from reagent kits or the environment where current sequencing technology and analysis [6–8] cannot reliably distinguish signal from noise. In contrast, the former pneumotype has consistently been identified by multiple investigators and has been associated with signs of lower respiratory tract inflammation, including increases in bronchoalveolar lavage (BAL) lymphocytes and neutrophils, elevated levels of exhaled nitric oxide, and enhanced Th17 expression [3, 4, 9]. In particular, the presence of high relative abundance of *Prevotella* and *Veillonella* has been associated with inflammation and various chronic lung diseases [10–13]. Studies of the virome have been more limited. Most prior studies have used targeted sequencing approaches to detect known viral pathogens such as rhinoviruses, parainfluenza viruses, and metapneumoviruses [14]. A recent study using shotgun sequencing to examine the virome in smokers and nonsmokers found predominantly bacteriophages [15].

Composition of the Post-Lung Transplantation Microbiome

In stable transplant patients, the most distinctive post-transplant community profiles appear to be primarily related to clinical features. An elegant study of lung transplant patients with cystic fibrosis (CF) found rapid recolonization of the graft by *Pseudomonas aeruginosa* from the upper airway reservoir [16]. However, *Pseudomonas*, along with *Acinetobacter,* has been overrepresented in the transplanted lung compared to the native lung in individuals with interstitial disease receiving a single lung transplant [17]. Longitudinal studies are important in following the progression of changes in the microbiota over time. One study demonstrated that the microbiota in the graft remain different for at least 1 year compared to the native lung in single-lung transplantation [17]. In a cohort of 12 individuals post-transplant followed over 3 years, a clear predominance of Proteobacteria was reported for all BAL samples regardless of clinical status [18].

Immunosuppressive drugs and antibiotics after transplantation are expected to pressure microbial communities to diverge from the non-transplanted healthy host [19–26], but an impact of the immunosuppressive regimen itself has not been demonstrated definitively [26, 27]. Antibiotic therapy does seem to impact the load, composition, and structure of bacterial communities [18, 19, 28, 29]. Clinically stable patients with physiologic inflammation and preserved respiratory function tend to have prevalent *Streptococcus*, *Prevotella*, and *Veillonella*; these dominant genera are accompanied by *Neisseria*, *Actinomyces*, *Rothia*, *Granulicatella,* and *Gemella* [10, 11, 28]. Of note, these genera frequently co-occur in unsupervised clustering based on composition. BAL samples from these individuals with intermediate alpha diversity, richness, and bacterial biomass have been shown to have culturable bacteria [28, 30, 31]. This profile mirrors that with supraglottic predominant taxa (pneumotype$_{SPT}$) from the studies of Segal et al. in healthy cohorts [3, 4]. These studies suggest that the major impact on microbes is due to graft-specific or alloimmunity-related factors, rather than the general context of immunosuppression or descending recolonization from the upper respiratory tract reservoir.

In post-transplant complications such as infection or acute rejection, the balanced pneumotype shifts to either (1) a profile with decreased alpha diversity, richness, and an increase in bacterial load with a predominance of typical opportunistic pathogens such as *Staphylococcus* and *Pseudomonas* or (2) a profile with a sharp decrease in bacterial load [28, 29]. In subsequent sections, we discuss specific relationships between post-transplantation complications and the respiratory bacterial microbiome.

Although the identification of microbiota profiles has proven useful in advancing our understanding of the clinical impact of host-microbe interactions after transplantation, the vast majority of compositional analyses performed to date have been based on 16S rRNA sequencing which lacks information on species and strain diversity. Studies that extend this approach through the use of culturomics, whole-genome sequencing, and phenotyping will make it possible to explore this diversity, notably the striking in the genus *Streptococcus,* where several different species typically coexist [28, 30, 31]. Shotgun sequencing of whole communities offers unique insights into understanding the microbiota and their functional impact on both the environment and the host. Using this technique, the acquisition of antibiotic resistance genes, especially those conferring multidrug resistance, was shown to correlate positively with bacterial diversity in BAL samples from pediatric cystic fibrosis transplant recipients [18].

Though less studied, fungi and viruses are present in the respiratory tract microbiome in lung transplant recipients. Studying fungi in the lung is comparatively harder than bacteria, and special care must be taken to analyze and interpret the results of internal transcribed spacer (ITS) sequencing [32]. In one study, *Candida* was most frequently identified in both the lower and upper respiratory tract, followed by *Aspergillus* which was seen in higher amounts in the lung [33]. Additionally, *Cryptococcus* was identified in a subset of samples. Impact of the fungal communities in lung transplantation is not well-understood.

Viral members of the microbiome may also be important. In the BAL of lung transplant recipients, the balanced pneumotype is associated with a moderate load of Anelloviridae, compared to the other three microbiota profiles associated with either low or high load [28]. This virus family, virtually ubiquitous in immunocompromised individuals, has potential to serve as a biomarker of host immune competence and is associated with the bacterial microbiota [34, 35]. In addition to the known virome, there is a significant amount of viral "dark matter" in the lung because of the limitations in viral databases that map reads from shotgun metagenomic studies. Despite these bioinformatic limitations, there have been advances in understanding novel viruses in the respiratory tract, and one such viral family of small, circular DNA virus was identified – named the Redondoviridae [36]. With time, there will certainly be a better understanding of the viral landscape and how these viruses interact with the host and other members of the microbiota in the lung. Overall, these observations of cross-kingdom associations highlight the need to consider the respiratory tract as an ecosystem, whose equilibrium is a prerequisite for preserved respiratory function.

HIV Respiratory Tract Microbiome

Given the increased susceptibility to pulmonary infections and the alterations in host immunity, HIV might be expected to impact the lung microbiome [37]. Somewhat surprisingly, the lung microbiome in people living with HIV (PLWH) is similar to healthy lungs when HIV is recently acquired or the disease is well-controlled on antiretroviral therapy (ART). In the largest cohort study to date, the Lung HIV Microbiome Project (LHMP) found that, in PLWH, there was no difference in BAL composition in either treatment-naive or those on ART compared to HIV-negative individuals [38][58]. The study also found that *T. whipplei* was increased in PLWH on ART but not in treatment-naive PLWH. An earlier study found that this organism was present in newly diagnosed HIV and decreased with initiation of ART. The significance of *T. whipplei* in the lungs is still incompletely understood, but it has also been detected in a non-human primate model of HIV [39].

Most studies of PLWH have focused on those with well-controlled or early HIV. It is possible that the microbiome would differ in more advanced HIV disease. A study of PLWH with advanced infection with a median baseline CD4+ count of 280 cells/uL found significant dysbiosis with reductions over time in richness (the number of taxa per sample) and their relative evenness (lower alpha diversity) compared to the HIV-negative group [40]. In addition, significant compositional differences as measured by UniFrac distances (further distances indicate less phylogenetic relatedness of the constituent bacteria) and altered taxa existed compared to HIV-negative controls. At baseline, there was an increase in *Streptococcus* and decrease in *Flavobacterium*, whereas after 1 year on ART, the BAL in PLWH had a notable increase in *Prevotella* and *Veillonella*.

Similar to lung transplantation, the fungal and viral components of the respiratory tract have been relatively understudied by culture-independent methods in PLWH. In a cohort study comparing PLWH and HIV-negative controls [41], the mycobiome in the lungs of PLWH showed increased fungal diversity and an enrichment in *Pneumocystis jirovecii*. When comparing oral washes to BAL, there was differential enrichment of *Ceriporia lacerata*, *Saccharomyces cerevisiae*, and *Penicillium brevicompactum.* Studies on the commensal virome using unbiased studies have been quite limited in PLWH. A study primarily analyzing the lung virome in transplantation used 3 individuals with HIV as controls and found that Anelloviridae were dominant [42]. Investigations using DNA and RNA preps of both blood and BAL found prevalent bacteriophage and evidence of actively replicating DNA viruses (such as Herpesviridae and Parvoviridae) as well as RNA viruses from the Picornaviridae and Flaviviridae families [43]. Interestingly, anelloviruses were found in blood but not lung samples.

Oral-Lung and Gut-Lung Axes

There has been emerging evidence that the oropharynx and gastrointestinal tract influence the lung microbiome. There are direct effects from anatomical contiguity of the oropharynx to the airways [2, 4, 44–46] and potentially gut-lung translocation of bacteria (mostly described in the context of critical illness [47] and severe immunosuppression [48]). There may also be indirect effects through shaping the microbiome via alterations in the inflammatory milieu and host response in the lung [49]. Given the alterations in the oral and gut microbiota composition and function in transplantation and HIV, it is important to consider the lung microbiome not in isolation but in conversation with other niches.

Lung Transplant, Aspiration, and Gut Dysbiosis

The oral microbiome may influence the lung microbiome in transplantation. Given that prior research indicates the main source of lung microbial communities is the oropharynx, the implication of this study is that before and after transplant the lung will be continually exposed to dysbiotic oropharyngeal communities. For example, lung transplant patients have suboptimal oral health [50] and also have a high incidence of gastric and oropharyngeal aspiration [51, 52]. An investigation of the oral microbiome before and after lung transplant found significant oropharyngeal dysbiosis in individuals with end-stage lung disease before transplantation [53]. They had a reduced diversity and increased facultative bacteria relative to aerobic bacteria and absent obligate aerobes in comparison to healthy controls. After transplantation, there was a partial normalization, but a return to dysbiosis by 6 months when the oral communities returned to the severe dysbiosis was seen in end-stage respiratory

disease. Other studies show that a subset of individuals after transplantation has an oral-type lung microbiome – i.e., the "balanced pneumotype." This relatively rich and diverse microbiota profile is seen in predominantly healthy subjects and stable transplant recipients.

In other solid organ transplants, the gut microbiome has been shown to be altered, but a clear understanding of the causal relations between the gut microbiome and transplantation outcomes is still in early stages [54, 55]. In the pediatric lung transplantation population, a study examined the oral, gastric, and lung bacterial communities in the context of gastric dysmotility [56]. Similar to the adult study above, they found marked decreases in alpha diversity in the oral and gastric niches. This effect was accentuated in patients with proven gastric dysmotility and proton-pump inhibitor and antibiotic exposures. In this study only 5 of 23 patients had enough DNA isolated from BAL so inferences about the gastric-lung and oral-lung axes were limited. Little work has been done on the lower GI tract in transplantation, but it is likely these microbes are altered in lung transplantation and may have systemic impact. Understanding the role of the oral-lung and gut-lung axes may open up an avenue of microbiome therapeutics in prevention or adjuncts to treatment of lung transplant complications [57].

The Relation Between the Oral and Gastrointestinal Tract with the Lung in HIV

In PLWH, there is evidence of a dysbiotic oral and gastrointestinal tract microbiome [58]. Key findings from multiple studies show that gut diversity is lower than healthy controls and that diversity correlates with peripheral CD4+ cell count. On a taxonomic level, there tends to be an enrichment of Proteobacteria and a higher ratio of *Prevotella* to *Bacteroides*. This altered gut microbial composition also drives systemic inflammation and even progression of HIV-related disease [58–61]. These studies show that in HIV with low CD4+ counts, the gut mucosal barrier becomes more permeable, and bacteria and/or bacterial components such as lipopolysaccharide (LPS) are able to translocate and drive inflammation that could impact lung function.

The oral microbiome also appears to be altered in HIV, perhaps more than the lung. A multi-center study found that there were differences in the oral microbiome with increased *Streptococcus* and *Actinomyces* in treatment naive PLWH, more *Rothia* in PLWH on ART, and *Atopobium* in both groups of PWLH compared to HIV negative [38]. Oral communities also seem to affect inflammation and lung function in PLWH [62]. Interestingly, no association was seen between the gut microbiome and lung function, or oral microbiome and lung function in persons without HIV infection. In this study, oral taxa correlated with systemic inflammatory markers TNF-alpha, MIP-1a, and endothelin-1; further, these systemic

inflammatory markers negatively correlated with the diffusing capacity for carbon monoxide (DLCO).

These data suggest both that the oropharyngeal microbiome is dysbiotic in PLWH and can therefore serve as a direct source to the lung and also that the oral microbiome sets systemic immune tone which can influence lung function. Therefore, in PLWH the oropharynx seems to have both direct and indirect effects on the host-microbiome relationship in the lung.

Mucosal Defenses

Tolerance, Immunity, and the Microbiome After Lung Transplantation

In immunocompetent individuals, the balance between host and microbes is based on the compartmentalization of the respiratory microbiota from the upper respiratory tract down to alveoli [63]. Along the respiratory tract, there are lymphoid tissue structures where complementary cellular and humoral responses are orchestrated. In particular, the production of type A and M immunoglobulins (Ig) plays an important role in conducting airways, while alveolar macrophages are involved in the respiratory zone (Fig. 10.1a). Depending on the compartment, the production of saliva, mucus, or surfactant-containing antimicrobial peptides complements these defenses. The transplanted lung also contains distinct phenotypic subsets of immature myeloid cells, with a predominance of immunosuppressive cells in the proximal airways, as opposed to a higher abundance of pro-inflammatory cells in the distal airways [64] (Fig. 10.1a). This study combined microbiome analysis of different fractions of BAL with flow cytometry to delineate the myeloid cells. They found the relative abundance of bacterial phyla associated with the two cell subsets was different.

A key triad in host immunity involved in the cross-talk with the microbiota for the maintenance of immune tolerance consists of innate lymphocytes (mainly gamma-delta (γδ) T cells, type 3 innate lymphoid cells (ILC3s), RORγt+ T-helper 17 (Th17), and Foxp3+ regulatory T cells (Treg), all of which are necessary for the production of lymphoid tissue such as bronchial associated lymphoid tissue (BALT) and IgA) [65] (Fig. 10.1b). γδ T cells and ILC3s, which infiltrate lung allografts and are important for maintaining tolerance in experimental models, are potent producers of interleukin-22 (IL-22), a necessary component for B-cell recruitment and BALT induction [66]. In parallel, IL-22 induces the production of antimicrobial peptides and mucins contributing to epithelial integrity, and IL-22-deficient mice have altered colonic microbiota, which induces colitis when transferred to wild-type mice [67]. Although an impact of IL-22 on the microbiota in the transplanted lung has not yet been revealed, the potential of this factor in the respiratory tract has been demonstrated in the mouse where *Candida albicans* airway exposure induced IL-22

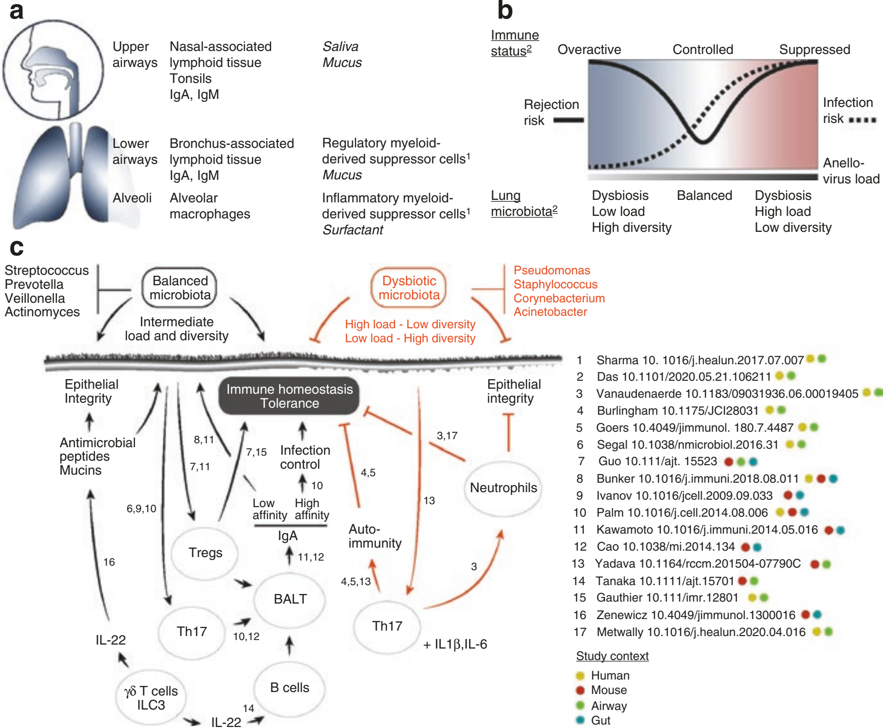

Fig. 10.1 Interactions between respiratory microbiota and host immunity in lung transplantation. (**a**) Schematic representation of the compartmentalization of mucosa-associated lymphoid tissue in the respiratory tract, with indication of the main inductive sites, humoral and cellular effectors, and mucosal secretions (italicized) involved. (**b**) Relationship between balanced or dysbiotic respiratory microbiota and immune tolerance, as reported in observational clinical studies, or inferred from preclinical studies, in the lung or gut, as indicated by a color code. (**c**) Synthetic diagram showing the risk of rejection (continuous line) and the risk of infection (dashed line) in relation to the immune status (blue-white-red gradient), the characteristics of the bacterial microbiota and the anellovirus load

release by innate lymphocytes which were protected against *Pseudomonas aeruginosa*-induced lung injury [68].

There is also a close link between the respiratory microbiota and Th21 cells and their cytokines IL-21A, IL-21F, IL-21, and IL-26. The microbiota profile enriched in supraglottic taxa is associated with increased levels of several cytokines relevant for Th21 cell differentiation (e.g., IL-1β and IL-6) and chemotaxis (fractalkine) [4]. These associations suggest that some of these bacteria play a role in the respiratory tract similar to that of segmented filamentous bacteria (SFB) in the gut [69] and contribute to the maintenance of a tonic level of immune monitoring (Fig. 10.1b). In turn, Th21 cells protect the surfaces of the respiratory mucosa against infection by bacteria and fungi [70]. However, activation of Th21 and Th17 cells under pro-inflammatory conditions such as those associated with dysbiosis triggers a vicious

cycle of neutrophil recruitment and autoimmune responses [71–73], with adverse consequences related to tissue damage, which ultimately breaks tolerance and increases the risk of chronic lung allograft dysfunction (CLAD) [74]. CLAD is a clinical syndrome of the chronic and progressive loss of allograft function after the first post-transplantation year and is the main driver of late morbidity and mortality [75, 76]. In support of this theory, a positive correlation was found between IL-21 levels in BAL and neutrophil counts on transbronchial biopsies during acute rejection, a significant risk factor for CLAD [77].

Similarly, CD4+ Foxp3+ regulatory T cells (Treg) lie at the interface between the microbiota and immune tolerance (Fig. 10.1b). Treg are primary cellular mediators of immune tolerance, and, accordingly, their abundance in the periphery is lower in CLAD versus stable recipients [72, 73]. Treg functions are altered by the main immunosuppressive drugs used in the context of lung transplantation, with an inhibitory effect by calcineurin inhibitors [74] and stimulation by corticosteroids [77]. No studies to date have demonstrated a direct impact of airway microbes on the expansion and differentiation of Treg, but there are converging indications that this may be the case. *Prevotella histicola*, a native commensal of mucosal surfaces, including in the mouth, nasopharynx, and gastrointestinal tract, induces the generation of Treg in the gut after enteral administration [78]. This bacterial species is taxonomically close to *Prevotella melaninogenica*, an important colonizer of the respiratory mucosa, most frequently present in a lung bacterial community associated with an anti-inflammatory profile after lung transplantation [28]. A remote effect of the microbiota was also observed in a mouse model of orthotopic lung transplantation, with the demonstration that the intestinal microbiota in the recipient plays a key role in controlling rejection [79]. Here, intestinal dysbiosis drastically altered the pulmonary and systemic levels of Treg, thereby increasing acute and chronic rejection [79]. A reverse impact of Treg on the microbiota has also been observed in the mouse gut, where Treg transfer by itself changes the composition of the microbiota and increases its diversity [80].

The formation of lymphoid tissue such as bronchus-associated lymphoid tissue (BALT) is an important node of convergence of innate lymphocytes, Th17 and Treg [66]. These cells stimulate production of polyreactive IgA that binds with low affinity to numerous microbial antigens, promoting microbiota diversification and mucosal homeostasis [66, 80–82] (Fig. 10.1b). Accordingly, in a mouse model of retransplantation, the early formation of Treg-rich BALT after lung transplantation promotes long-term allograft tolerance [83]. In contrast, treatment of recipients with an anti-CD25 antibody that depletes Treg abrogates tolerance [83].

Although the evidence presented above linking immune monitoring, microbiota, and immune tolerance is based on associations demonstrated by observational studies or on data obtained from animal models that do not include the immunosuppression used in the context of human lung transplantation, it supports a link between balanced airway microbiota, with preserved diversity, and a clinically stable post-transplant state [28] (Fig. 10.1c). This association favorable to long-term graft survival differs from two other conditions linked to distinct types of dysbiosis and post-transplant complications related to infection and rejection [28]. As studies in

the transplanted lung have failed so far to show a direct link between the level of immunosuppressants administered and respiratory microbiota features, it appears that the net immunocompetence level of transplant recipients should be considered, which varies according to multiple parameters including immunosuppression and current alloimmunity status. In this respect, the monitoring of anellovirus levels in the peripheral blood or lung, where it is aligned with the bacterial microbiota profile and gene expression pattern of BAL cells, provides a useful tool for determining net host immunocompetence [28].

Overall, the respiratory tract microbiome is an essential factor in setting the immune setpoint in the lung through multipronged interactions after lung transplantation. Further work needs to be done elucidating how these networks are operating in parallel, what are some of the feedback loops involved, what are the key elements of the microbiome that regulate immune responses, the evolution over time, and finally how the interactions promote alloimmunity or tolerance.

HIV and Epithelial Function, T-Cell Exhaustion, and Trained Immunity

While persons undergoing lung transplantation and PLWH are both considered immunosuppressed, there are significant differences between them. Lung transplantation disrupts physical barriers, results in repeated stimulation by pathogen-associated molecular patterns (PAMPs) and tissue damage-associated molecular patterns (DAMPs) that activate innate immunity and lead to anti-donor alloimmunity, and leads to disrupted adaptive immunity [75, 84]. In addition, a multi-drug immunosuppressive regimen is necessary for induction and maintenance of immune tolerance of the allograft [85]. In contrast, HIV is primarily a viral-induced depletion of helper T cells and a consequent state of non-specific immune activation, detailed below [86].

The lung is a known reservoir for HIV infection, and direct HIV infection of bronchial epithelial cells alters the critical functions of the airway epithelium [87–89]. The HIV *trans*-activator of transcription (Tat) protein suppresses cystic fibrosis transmembrane receptor (CFTR) transcription via transforming growth factor (TGF)-beta signaling [89]. CFTR is dysregulated in other lung diseases such as COPD and may play a role in shaping the lung microbiome in HIV [90]. Bacterial sphingomyelinase, which is produced by *Staphylococcus* among other bacteria, inhibits the CFTR [91]. A study of untargeted metabolomics of exhaled breath condensate both before and after pulmonary exacerbations in CF found multiple metabolites of microbial origin [92]. Therefore, there appear to be two hits to CFTR function in PLWH through an altered microbiome and direct HIV infection which could be a pathogenic mechanism underlying impaired mucus physiology in the airways of PLWH. COPD pathogenesis in PLWH is discussed in further detail below.

Another core function of epithelial cells (specifically the type II cell) is the production of surfactants, and there is emerging data suggesting that surfactant physiology is altered in HIV infection [37, 93]. These are lipid-protein complexes that, aside from their critical role in maintaining alveolar architecture, modulate the response to infection [94]. In addition, their lipid/protein complex structure allows them to act as a sink to prevent LPS or viruses from interacting with the epithelium and invoking an inflammatory response. They also have innate antimicrobial properties. Surfactants promote microbial aggregation and opsonization which improves immune clearance of potential pathogens in the lower respiratory tract. After lung injury, they help to promote tissue recovery by modulating apoptosis, efferocytosis, and promotion of regeneration.

Airway microbiota have bidirectional relationships with surfactant protein secretion. Surfactant protein D (SP-D) promotes resistance to HIV infection [95, 96]. In a murine model of influenza infection, nasal pre-challenge with *Bifidobacterium longum* increased both SP-D and interferon-gamma which had a protective effect from subsequent influenza infection [97]. In the gut, SP-D that is produced in the gallbladder and secreted into the gut lumen influenced the composition of the commensal microbiota [98]. Therefore, HIV leads to lung epithelial dysfunction through direct infection and may alter CFTR and surfactant biology, and there is evidence that microbes may influence this dynamic.

There has been a comprehensive recent review of the mucosal immunology of the HIV respiratory tract and the intersection with the microbiome [37]. Macrophage and natural killer (NK) cell function appear to be dysregulated by HIV. Direct infection of dendritic cells (DC) leads to chronic T-cell activation and T-cell exhaustion. Chronic antigenic stimulation and activation of T cells leads to exhaustion in which cells are less able to respond to an infectious insult [99]. These cells have upregulated PD-1 and CTLA-4 receptors on the cell surface. In the lungs of PLWH with chronic, advanced disease, there is a lymphocytic alveolitis with low CD4/CD8 ratios. In addition, there tends to be an altered cytokine profile skewing toward a Th2-predominant response. In B-cells, chronic HIV leads to chronic activation and non-specific hypergammaglobulinemia. More recently, the intersection of innate and adaptive immunity has been of interest as it has become clear that these two arms of the immune system are not so neatly divided. The γδ-T-cells and ILC3s described above function as intermediaries between the two canonical arms of the immune system.

Microbes may also influence trained immunity, whereby a microbial response induces functional and lasting innate immune memory, generally on the scale of months to years, that facilitates clearance of future pathogens and enhances responses to sterile insults [100]. Figure 10.2 summarizes the putative ways airway dysbiosis might influence trained immunity. In many ways, trained immunity is the opposite of tolerance. Whereby tolerance is the process of dampening the innate immune response to repeated antigenic exposure, which is central to allograft tolerance after transplantation, trained immunity is an enhanced and more efficient

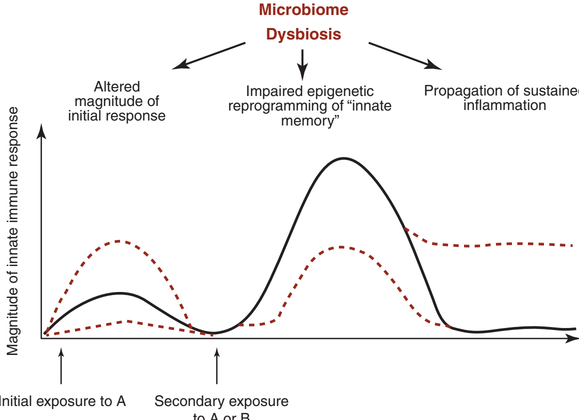

Fig. 10.2 Microbiome and trained immunity. Trained immunity is an augmented innate immune response to a subsequent exposure to a pathogen or a broad immune protection to a range of potential pathogens after an initial insult, leading to resolution of infection (black line) [100]. Microbial dysbiosis may disrupt this enhanced protection (red dotted line) through a number of mechanisms. First, airway dysbiosis can cause a pathologically high or low initial innate response [49, 91, 101]. Second, trained immunity relies on "innate memory" formation through a complex process of epigenetic and metabolic changes. In the gut and vagina, dysbiosis has been shown to alter this "innate memory" process [102, 105]. Third, dysbiosis can lead to a state of chronic innate immune stimulation and an inhibition of inflammation resolution [28, 37, 186]

innate immune response to a future challenge of the same or a heterologous pathogen-associated molecular pattern (PAMP). An example of trained immunity is from a murine preclinical model of adenovirus lung infection where the infection induced memory in alveolar macrophage. This memory function was dependent on IFN-gamma signaling from T-cells [101]. Subsequently, these mucosal memory alveolar macrophages conferred increased protection against bacterial pathogens such as *Streptococcus pneumoniae*. In HIV, trained immunity is seen in the resistance to acquiring HIV infection based in part on vaginal microbiome composition that alters mucosal defenses [102]. In this setting, dysbiotic states (which interestingly in the vaginal microbiome are higher diversity communities) are pro-inflammatory, recruit inflammatory cells including CD4+ cells, and increase risk of HIV acquisition. By contrast, communities dominated by the commensal *Lactobacillus* offer protection against HIV acquisition across the vaginal mucosa. Mechanistic work has pointed to lactic acid produced by lactobacilli as decreasing the production of pro-inflammatory cytokines interleukin-6 (IL-6), IL-8, tumor necrosis factor-alpha (TNFa), chemokine ligand 5 (CCL5 or RANTES), and macrophage-inflammatory protein 3a (MIP3a) from vaginal epithelial cells in response to TLR-agonists [103, 104]. Finally, there has been interest in harnessing trained immunity through vaccines to prevent HIV acquisition. A study of simian-human immunodeficiency virus (SHIV) challenge in macaques found that low ratios of *Bacteroides/Prevotella* and low Firmicutes led to high rectal CD4+ cells in the mucosa and greater susceptibility to infection [105].

The above discussion highlights the complex impact of HIV and lung transplantation on the interactions between the host and the microbiome. Despite the complexity of understanding host-microbiome interactions at the mucosal interface in immunosuppressed states, dissecting these relationships and their underlying mechanisms is a promising frontier to understanding complications that arise from lung transplantation and HIV. Next, we explore how the intersection of immunity and metabolism helps to shed light on these relationships.

Immunometabolism

There has been increased interest in understanding how host-microbe interactions help set basal immune tone, maintain epithelial barrier integrity, and also contribute to disease when disruptions in the balance occur. Metabolism sits squarely at these interfaces. Microbes elaborate metabolites such as short chain fatty acids, secondary bile acids, and nicotinic acid that influence both immune and epithelial function [106]. Therefore, studying the metabolome holds promise to understand microbial metabolites that influence immune function and changes in host metabolism that may precede disease and to identify predictive and prognostic biomarkers as well as putative therapeutic targets [107]. Figure 10.3 outlines the uses of metabolomics in understanding host-microbiome relationships in the context of lung health and disease.

Lung Metabolome After Transplantation

The metabolome of the lung after transplantation may be influenced by the microbiome as well as by post-operative antibiotics and immunomodulators. In a study of six adults with interstitial lung disease who received a single lung transplant, BAL collected from the allograft and native lung was analyzed by liquid chromatography-high-resolution mass spectrometry [17]. Profiling of 16S rRNA and cytokine levels were determined in parallel. There were higher levels of the host-derived metabolite acetylated proline-glycine-proline (Ac-PGP) in the allograft compared to the native lung. This tripeptide is derived from collagen after multiple proteolysis steps, including the action of metalloproteinases (MMP)-8 and MMP-9, and acts as a strong neutrophil chemoattractant [108]. Correspondingly, the transplanted lung had higher bacterial biomass and higher relative abundance of the genera *Pseudomonas* and *Acinetobacter* [17], classical opportunistic bacteria whose proliferation is associated with pulmonary neutrophilia, MMP activation, and impaired immune tolerance [10, 29, 109]. In addition, the allograft contained lower levels of sphingosine, a lipid capable of killing many bacterial species, including *P. aeruginosa*, *Staphylococcus aureus*, and *Acinetobacter baumannii* [110], further strengthening the links between the respiratory microbiota and metabolome.

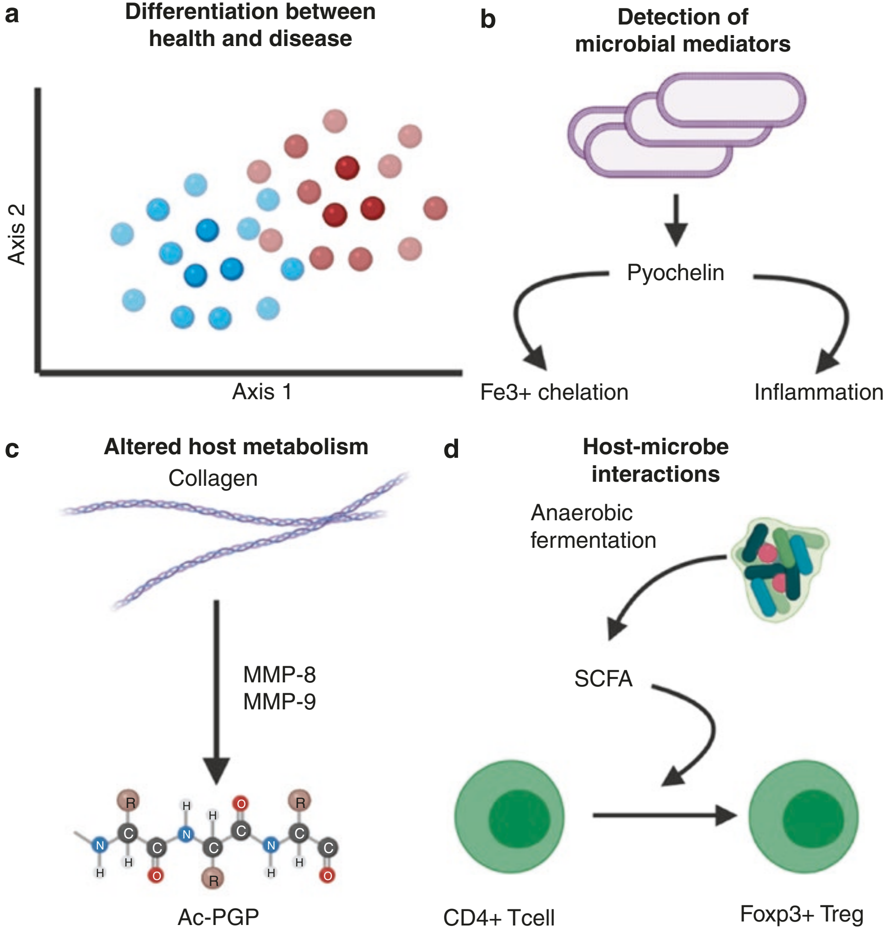

Fig. 10.3 Metabolomics and host-microbiome interactions in the lung. (**a**) The use of metabolomics distinguishes between health and disease states in both HIV and lung transplantation [17, 112, 113]. (**b**) Metabolomics can identify known microbial virulence factors [112] or (**c**) alterations in host metabolism [17]. (**d**) Additionally, metabolomics can help shape our understanding of the host-microbe interface and its relation with disease states [115]

HIV Lung Metabolome

Metabolomics has similarly been used to functionally profile the lung in PLWH. There has been an interest in understanding why PLWH have an increased susceptibility to lung disease such as pneumonia, tuberculosis (TB), and COPD despite effective ART and low viral loads [111]. Cribbs et al. investigated

untargeted metabolomics in BAL from asymptomatic PLWH (*n* = 24) on ART with a median CD4+ count of 432 cell/uL and healthy controls (*n* = 24) [112]. They found that the metabolomic signature could effectively discriminate between individuals based on their respective HIV status suggesting that despite relatively normal CD4 counts, there are functional metabolic differences at baseline. Interestingly, many of the metabolites identified by a unique *m/z* mass-to-charge ratio did not map back to a known metabolite raising the prospect that they were microbially derived or perhaps novel metabolic pathways. One such metabolite increased in PLWH was a pseudomonal siderophore named pyochelin, a compound that chelates iron.

A follow-up study analyzed the associations between CD4+ count, the lung microbiome by 16S rRNA sequencing, and the lung metabolome between PLHW and healthy volunteers [113]. Although the bacterial microbiome did not differ by HIV status, the metabolic profiles of the lungs distinguished PLWH from controls and were associated with particular bacteria. The metabolome correlated with the peripheral CD4 count, and four metabolites were enriched in the PLWH group – cystine, a tyrosine derivative, and two complex carbohydrates. Cystine is particularly notable as it is the oxidized form of cysteine, and the lung after HIV is thought to be in an altered redox state. When the relationship between the lung microbiome and metabolome was analyzed, there were three bacterial families (*Staphylococcaceae*, *Caulobacteraceae*, and *Nocardioidaceae*) and one genus (*Streptococcus*) highly correlated with the metabolome. Lineolate, glycerophospholipid, and fatty acid metabolism were the metabolic pathways most highly correlated with the microbiome. This study suggests complex and relevant relationships between the microbial composition of the lung in HIV and the metabolic environment, but additional studies are needed to understand the implications of these findings.

The metabolome in HIV may also be altered during lung bacterial infection and tuberculosis. The specific disease associations are detailed in the next section. In the setting of PLWH with pneumonia in a Ugandan cohort, distinct lung microbial communities are associated with local immune response and metabolomic signature in the circulation [114]. A separate study examining PLWH and tuberculosis found a relationship between *Prevotella*, short chain fatty acid (SCFA), and risk of tuberculosis [115].

Taken together, the body of work in lung transplantation and HIV show the close links between microbial communities, host physiology, and metabolism in the respiratory microenvironment. These interactions have important consequences for immune tolerance, long-term transplant outcomes, and lung disease following HIV despite adequate ART and involve cellular and humoral factors of host immunity. Specific examples of the dysregulated host-microbe interface are discussed in detail in the following section.

Microbiota-Mediated Diseases: PGD, CLAD, Lung Infection, and Emphysema

Primary Graft Dysfunction (PGD)

PGD occurs within 72 hours after transplantation and results in severe lung injury. It is the leading early cause of death post-transplant and a predictor of late complications [116]. Independent studies have shown that an early increase in the load of alphatorquevirus (anellovirus family) through the first week after transplantation is associated with a lower risk of developing severe PGD [34, 117]. This finding suggests that immune activation or tissue damage related to PGD influences the microbiota, notably restricting viral replication. Bacterial communities have not been examined in these studies, but correlations between anellovirus and bacterial communities are known in the respiratory tract post-transplant, with abnormally low or high viral loads correlating with bacterial dysbiosis [28, 42] (Fig. 10.1c). The first months after transplantation represent the period most at risk for the development of infection. Infectious episodes, generally limited in time, are often due to an established member of the microbiota, for which the decrease in immune surveillance opens the door to proliferation and acquisition of virulence factors. From a pulmonary ecology point of view, the disturbance due to infection is typically accompanied by additional perturbations from the prescription of antibiotics [118]. However, the pulmonary microbiota show a high resilience capacity during this period [28]. An examination of oropharyngeal lavage samples obtained 6 weeks, 3 months, and 6 months after transplantation, or before transplantation, showed partial normalization of the microbiota during the first 3 months post-transplant, but the subsequent appearance of severe dysbiosis is similar to that of patients before transplantation [53].

CLAD

CLAD is defined as a sustained 20% or more decrease in FEV1 or FVC. It has an obstructive form known as bronchiolitis obliterans syndrome (BOS) and a restrictive form known as the restrictive allograft syndrome (RAS). CLAD has complex and incompletely understood pathogenesis that has a final common pathway of airway fibroproliferation in BOS and peripheral and pleural fibrosis in RAS. The only treatment shown to slow the progression of disease has been azithromycin [119], and multiple clinically recognized potential pathogens such as cytomegalovirus [120], community-associated respiratory viruses [121], *Pseudomonas* [122–124], and potentially *Aspergillus* [125, 126] increase the risk of CLAD. In addition, a body of work suggests those with chronic aspiration are at high risk of acute rejection and CLAD [127, 128].*Therefore, the respiratory tract microbiome and its relation to CLAD has been an area of active investigation.*

Longitudinal analyses may shed light on the relationship of the microbiome to CLAD. The use of MetaLonDA, a bioinformatics tool dedicated to longitudinal differential analysis [129], provided interesting lessons on the follow-up of 12 lung transplant recipients over 3 years, with 5 patients developing CLAD during the study period [18]. While bacterial diversity was comparable between the CLAD and stable groups in aggregate, consideration of post-transplant time revealed divergent patterns. The stable group had an increase in diversity between the time of transplantation and the end of the study period, and, in contrast, bacterial diversity was significantly reduced in the CLAD group starting from 10 months post-transplant. Of note, this time point preceded the histopathological diagnosis of CLAD in 4 out of 5 patients, suggesting that bacterial diversity may serve as a predictive variable for CLAD development.

Other studies have sought to determine relationships between CLAD and the bacterial microbiome though significant differences in study design and methodology preclude any clear relationship between specific taxa or microbiome diversity metrics and CLAD. In CF, the presence of *Pseudomonas* spp. post-transplant was protective against bronchiolitis obliterans syndrome (BOS), the main cause of CLAD, particularly if the patient had *Pseudomonas* identified by culture pre-transplant [12]. In contrast, enrichment with *Streptococcus* and *Veillonella* and decreased diversity over time within an individual was associated with increased BOS incidence. These associations did not hold for non-CF patients. Adding further complexity, both time- and location specificity matter in changes in the microbiome associated with CLAD. One analysis found Actinobacteria dominance at 3 months post-transplant was associated with reduced rates of BO*S*, less inflammation in BAL, and lower rates of acute cellular rejection [130]. However, this association did not hold for later time points. In an examination of topographical features of the post-transplant lung microbiome [64], there were different associations between BOS and the microbiome that were dependent on the localization along the airway. The most recent study, which was prospective and rigorously done, analyzed BAL from 134 subjects 1 year after transplantation and analyzed predictors of subsequent composite outcome of death or CLAD that develops within 500 days after sampling [131]. They found that bacterial biomass was positively correlated with the composite outcome, even when controlling for demographic and clinical factors including immunosuppression.

Taken together, these observations suggest that after an initial period of a few months associated with a preserved state outside of episodes of acute disturbance, the microbiota of the transplanted lung tend toward dysbiosis preceding the histopathological diagnosis of CLAD. It is tempting to think that microbial dysbiosis contributes to the chronic inflammation, the consequent aberrant tissue remodeling, and fibrosis typical in CLAD [75]; however, it is difficult to disentangle cause and effect. Presumably, this is a feed-forward loop between an increasingly dysbiotic microbiota and reciprocal amplification of inflammation and tissue remodeling; the latter then pressures the evolution of microbiota composition. More experimental and translational research with a longitudinal and interventional design is needed to explore the causal links between immunosuppression, immune surveillance, alloimmunity, airway microbiota dysbiosis, and CLAD.

Pneumonia and TB

Dysbiosis of the respiratory tract may result from or lead to acute pulmonary infections. A Ugandan cohort of PLWH (median CD4+ of 71 cells/uL) and pneumonia (n = 182) had BAL 16S rRNA, BAL immune responses using qPCR on RNA extracts, and the serum metabolome examined [114]. The bacterial microbiome had three main clusters each with a different predicted metagenomic function using imputed metagenomic analysis (PICRUSt), and these clusters were associated with clinical outcomes. The cluster characterized by *Pseudomonadaceae* had the highest prevalence of positive mycobacterial cultures, the lowest 70-day mortality at 13% (though this mortality was not statistically different than the other 2 groups); the *Streptococcaceae* cluster had a 16% mortality; and *Prevotellaceae* had the most Aspergillus culture positivity and had the highest mortality at 22%. The *Pseudomonadaceae* cluster was associated with high T-cell immunoglobulin and mucin domain (TIM)-3 expression. This cluster was associated with the highest peripheral levels of tryptophan, arachidonic acid, and secondary bile acid metabolites. The *Streptococcaceae* group had low TIM-3 and elevated protein-arginine deiminase type-4 (PADI4) and IL-10. The *Prevotellaceae* cluster had an induction of IFN-alpha and Th2 responses; however, they had lower serum inflammatory metabolites, but higher valine and leucine metabolites, purine products. Overall, this study showed that lung microbiome composition was linked to a unique serum metabolomic signature, and these correlated with clinical outcomes in pneumonia.

The metabolic products of the lung microbiome may also influence susceptibility to tuberculosis. It has been known that lung lymphocytes from HIV-infected individuals on ART release less IFN-gamma when challenged with TB antigen [132]. Additionally, the gut microbiome produces short chain fatty acids (SCFAs) through anaerobic fermentation, and SCFAs drive CD4+ cells toward an anti-inflammatory Treg phenotype [133]. This anti-inflammatory phenotype is beneficial in maintaining mucosal homeostasis, but might compromise the ability to control TB. Therefore, Segal et al. sought to clarify this axis in a cohort of South African patients with HIV on ART who were at risk of TB disease [115]. They found that PLWH had higher levels of serum SCFAs compared to healthy controls. They also found that lower IFN-gamma, IL-17A, and higher butyrate and propionate (both SCFAs) in the serum at a baseline predicted development of TB disease. In the lung, the relative abundance of *Prevotella* was correlated with higher SCFA levels. The higher SCFA levels were positively correlated with TB antigen-induced Tregs and negatively correlated with IFN-gamma and IL-17a. These findings suggest that *Prevotella*-induced SCFA production leads to a hypo-inflammatory response and decreased ability to immunologically control TB in the lung.

COPD and HIV

There have been numerous studies linking the composition of the microbiome to COPD in the HIV-uninfected population [134]. In early COPD and in smokers with mild disease, there is very little, if any difference, in the lung microbiome

composition compared to health [1, 135, 136]; however, there has been a link between oral taxa (such as *Veillonella* and *Prevotella*) and increased BAL lymphocytosis and neutrophilia. There is abnormal composition in more advanced disease and exacerbations with Proteobacteria dominance, most notably *Haemophilus* that correlated with neutrophilia and *Moraxella* that correlated with interferon signaling [137]. The sputum microbiome composition during an acute exacerbation with low alpha diversity and higher abundance of *Staphylococcus* predicted higher 1-year mortality [138].

Even in the era of effective ART, the risk of COPD appears to be elevated compared to HIV-negative comparison groups, even after controlling for tobacco smoking [139]. A low DLCO is the most characteristic finding in PLWH and associated with an emphysema phenotype, airway obstruction, and pulmonary vascular disease, suggesting that the decrease in DLCO in HIV may be multifactorial [140–143]. The epidemiological findings suggest a unique phenotype of COPD in PLWH, and changes in the microbiome have been postulated to play a role in COPD in this population. In addition, accelerated aging, altered innate defenses, high systemic inflammation, abnormal alveolar macrophage function, altered redox state, and skewed protease/antiprotease ratios may all contribute to lung disease in this population and may impact the microbiome [37, 93, 144].

Despite the hypothesized impact of the microbiome on HIV-related COPD, relatively few differences have been detected using bacterial 16S rRNA sequencing in lung samples. One small, uncontrolled study of bronchial brushes in PLWH ($n = 21$) found only subtle differences in the bacterial microbiome between those with COPD by spirometry or radiographic criteria [145]. The authors did note that Firmicutes presence and low diversity tended to influence the bronchial cell transcriptome with a negative relationship with cilia-related genes and positive with immune response genes. A follow-up study by the same group included a control group and again used bronchial brushes to compare PLWH ($n = 28$) to HIV-negative controls ($n = 48$) [146]. While the study found differences in the microbiome alpha- and beta-diversity, as well as taxonomic composition between HIV+ and HIV- groups, it did not identify differences in diversity or composition based on COPD status both within and between groups. The detection of *T. whipplei* in the lung in HIV was postulated to play a role in HIV-associated COPD as it was detected more commonly in smokers [147]. *T. whipplei* has not been independently associated with lung function or with inflammation, making it unlikely that this bacteria plays a significant role in COPD [145, 146]. Additional studies with larger sample size and analyses of bacterial function and not just taxa may yet detect differences in the bacterial microbiome between COPD+ and COPD- groups in PLWH.

The oral and gut microbiota may also contribute to COPD in HIV. A study comparing saliva in PLWH and HIV-negative individuals found that the composition of the oral microbiome differed by both HIV status and smoking [62]. Alpha diversity was reduced in PLWH who were smokers. PLWH who had impaired DLCO had decreased alpha diversity and increases in relative abundance of *Veillonella*, *Streptococcus*, and *Lactobacillus*. This study did not find a relationship between gut microbiome and lung function in HIV. In other studies, low diversity of the gut microbiome in PLWH was linked to low CD4+ counts, high monocyte activation,

impaired gut mucosal integrity [148], and high IL-6 [61]. In the study of Vujkovic-Cvijin et al., the dysbiotic gut microbiota dysregulated the kynurenine pathway of tryptophan metabolism and higher systemic IL-6. This finding suggests a potential link of the metabolic function of the gut microbiome with COPD in HIV as higher peripheral IL-6 levels are associated with lower FEV1, FVC, and diffusion [149].

Fungi in the respiratory tract may also play a role in COPD in PLWH. In one study, 37% of patients with advanced COPD (GOLD stage IV) had evidence of *Pneumocystis* colonization [150], and in an autopsy study, 46% of PLWH had evidence of colonization in lung tissue [151]. In a non-human primate model of SIV infection, colonization with *Pneumocystis jirovecii* led to increased inflammation, bronchus-associated lymphoid tissue (BALT) formation, and subsequent emphysema [152]. An analysis using culture-independent ITS and 18S amplicon sequencing of BAL again noted an overrepresentation of *Pneumocystis* in the BAL of PLWH and COPD [41]. However, despite consistent findings across species, time, and methodologies showing the presence of *Pneumocystis* in the lungs of individuals with HIV/SIV infection and COPD, an interventional study that treated SHIV-infected monkeys with *Pneumocystis* colonization with trimethoprim-sulfamethoxazole did not prevent progressive obstructive disease [153]. This lack of effect may result from the time-sensitivity of fungal colonization, with early exposure leading to irreversible pathobiological changes that do not respond to later treatment.

A major limitation of studies in HIV and COPD is the focus on Western Populations [154]. Worldwide, the epidemiology of COPD has a notable influence from non-smoking causes – namely biomass fuel smoke [155] and tuberculosis [156]. Biomass fuel smoke disproportionately affects women and children exposed to cooking without ventilation in enclosed spaces. PM2.5 exposure impairs lung host defenses, increases risk of pneumonia, and exacerbates chronic lung disease [157]. One study in China found interrelation between PM2.5 exposure, the airway microbiome, and lung function [158]. Other causes include pesticides which lead to an increased incidence of fixed airway obstruction among the world's farming population [159]. These observations and early studies suggest a complex interaction between the place one resides, socioeconomic status, and the resultant environmental exposures that influence the microbiome and subsequently lung function.

Inflammation and Aging

Many pulmonary complications in individuals with lung transplants or with HIV are associated with chronic inflammation and resemble diseases observed in a normal aging population. This section will focus on the role the microbiome may have in normal lung aging and diseases associated with aging.

One common thread frequently found in normal aging as well as in age-associated chronic diseases is low-level inflammation [160], an observation which has been extended into the lung compartment [161, 162]. This inflammation has been

associated with development and/or accelerated progression of age-related chronic diseases [163–165]. Sources of low-level inflammation in aging remain poorly understood but are likely to include an altered interplay between the aging immune system and environmental microorganisms over the lifespan. This interaction can result through interface of lung cells with microbes through PAMPs resulting in release of innate inflammatory mediators [166]. Alternatively, repeated stimulation of cellular immune responses can result in an "immunosenescent" state, characterized by an accumulation of highly differentiated effector lymphocytes which spontaneously secrete inflammatory mediators, especially IL-1, IL-6, and TNF-α [167–170]. Many chronic diseases have been associated with an increased frequency of terminally differentiated lymphocytes, including chronic lung disease [171], cancer [169], and autoimmune diseases such as rheumatoid arthritis [172]. Lower airway microbes can also have significant effects on the lung metabolic profile, shifting it toward a more inflammatory state [4, 113, 173]. Thus, the lung microbiome has significant potential to define the lower respiratory tract inflammatory milieu.

There are many studies linking lung microbiome dysbiosis to lung diseases, many of which are found in an aging population. For example, advanced emphysema has been linked to increased Proteobacteria in lung tissue [174]. Two separate studies found increased V*eillonella* in the lungs of patients with lung cancer [175, 176]. In contrast, there are virtually no studies that directly address whether normal aging results in changes in the lung microbiome. The only study to date found increased Firmicutes and decreased Proteobacteria in sputum from an older Asian population compared to younger subjects and that this change was associated with increased peripheral arterial stiffness [177]. Studies on the changes in the lung microbiome with age are desperately needed.

Viruses, by their ability to establish latency, are thought to be the most likely cause of persistent antigenic stimulation [178, 179]. During the lifespan, older adults acquire numerous persistent infections that have the potential to repeatedly stimulate both innate and adaptive immunity and perpetrate inflammation. In addition to a lifetime of accumulating viral infections, the viral burden could be higher in older individuals due to a waning immunity with aging, creating a greater opportunity for latent viruses to reactivate.

Studies examining the virome in the normal lung are sparse, and the area is currently under intense investigation. The Twigg research group has recently completed a detailed virome analysis in PLWH and a control population (manuscript submitted). Using unbiased deep (average 60 million reads) shotgun sequencing on both host DNA and RNA from bronchoalveolar lavage cells, the study identified numerous viruses in the lower tract of healthy individuals. In addition to the bacteriophages and annelloviruses, multiple human endogenous retroviruses and various herpesviruses (especially HHV-4 and HHV-6) were found in 20–40% of healthy individuals. Herpesviruses were also detected in RNA preparations, implying low level replication was taking place since herpesviruses are DNA viruses.

The finding of herpesviruses in the lower respiratory tract has significant implications for the development of chronic lung disease. Numerous reports have linked

herpesviruses to chronic diseases of aging, including dementia [180, 181]. Both HHV-4 and HHV6 have been linked to pulmonary fibrosis, a common disease in the aging population [182]. Besides the known relationship of HHV-8 with Kaposi's sarcoma [183], there are associations of HHV-6 with other lung cancers [184]. Even progression of chronic emphysema has been linked to multiple viruses independent of their ability to induce acute exacerbations [185]. These observations lead to speculation of whether we should be more aggressive in eliminating chronic viral infections, especially those associated with latency.

In summary, there is ample evidence that the lower respiratory microbiome (both bacteria and viruses) can influence the inflammatory milieu, either positively or negatively. Likewise, there is similar data to support the paradigm that most of the lung diseases seen with aging are associated with chronic inflammation. Whether there is a direct relationship between the lung microbial milieu and chronic lung disease warrants further intensive research, including whether the ability to alter the pulmonary microbial environmental can ameliorate the mortality and morbidity of lung disease seen in the aging population.

Conclusion

In this chapter, we highlighted the complex relationships between the host and microbiome that exist in the immunocompromised states of HIV infection and lung transplantation. While there are emerging links between microbial diversity and composition, immune responses, and clinical outcomes, more work is needed to determine directionality and causality. Finally, although lung transplantation and HIV infection are both immunocompromised states, attention to the specifics of the immune deficits and the impact of concomitant treatments and exposures is needed to better understand the role of the microbiome in disease development and opportunities for new therapeutic directions.

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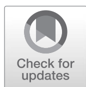
Chapter 11
The Microbiome in Acute Lung Injury and ARDS



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Acute Lung Injury and the Clinical Syndrome of ARDS

Originally described by Ashbaugh and colleagues in 1967 [1], the acute respiratory distress syndrome (ARDS) remains a serious threat to patients and a management challenge for critical care practitioners to this day. Defined as the acute onset of profound hypoxemia in the context of a predisposing insult and associated with bilateral opacities on radiographic imaging according to the Berlin definition [2], ARDS affects up to 10% of patients admitted to an intensive care unit (ICU), with a mortality of ~35% and long-term consequences in survivors [3]. In 2020, healthcare systems across the globe witnessed an unprecedented – in volume and time concentration – presentation of patients with ARDS due to pneumonia caused by the novel pathogen severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [4]. Conservative estimates for the incidence of ARDS in the USA during 2020 due to the new coronavirus disease (COVID-19) predict that at least 600,000 patients were hospitalized with ARDS [5], more than tripling the annual incidence of ~190,000 patients with ARDS prior to 2020 [3, 6].

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ARDS can develop as an inflammatory lung injury following insults directly impacting the lung epithelium, such as pneumonia, aspiration, and inhalational injury, or in response to systemic insults (indirect lung injury), such as sepsis, severe trauma, pancreatitis, and massive transfusion, among others [2, 3, 7]. The central pathogenetic feature of ARDS is disruption of the epithelial-endothelial interface and subsequent alveolar filling with permeability edema [8]. The corresponding histopathological features of acute lung injury (ALI) involve interstitial and alveolar edema with hyaline membrane deposition (diffuse alveolar damage – DAD), as well as alveolar hemorrhage, influx of neutrophils, fibrin deposition, alveolar collapse, and formation of microthrombi. Notably, only about half of patients clinically diagnosed with ARDS have been found to have DAD on post-mortem examinations [9], and the inter-provider reliability of ARDS diagnosis has been limited [10]. Therefore, ARDS is a clinically and biologically heterogeneous syndrome, encompassing a wide range of etiologies, severity of illness, and pathophysiologic mechanisms.

To date, treatment has mainly focused on supportive care while attempting to reverse the predisposing condition. Indeed, only the application of a lung protective ventilation strategy [11] and early prone positioning in severe cases have been demonstrated to improve mortality [12]. More recently, insights into biological endotypes of ARDS have been obtained, with the discovery of two distinct subgroups (so-called hyper- vs. hypo-inflammatory subphenotypes) that behave differently with respect to outcomes and responses to therapeutic interventions [13–17]. While the features defining these subphenotypes are well-characterized, the pathophysiological mechanisms underlying their distinction remain poorly understood. Recent evidence suggests that lung microbial communities may contribute to variability in host-responses and clinical outcomes in mechanically ventilated patients with acute respiratory failure [18, 19]. In this chapter, we explore the pathophysiological role of the lung and gut microbiome in the broader context of ALI/ARDS with an eye toward potential therapeutic interventions to improve outcomes.

The Microbiome Hypothesis in ARDS

The human microbiome in critical illness has been proposed as the "*forgotten organ in multi-organ failure*" [20]. Conceptualized as an internal organ, a disrupted microbiome can harm the critically ill host, similar to other "organ failures" in the ICU: damage that can be caused by both the "organ" function being lost as well as the aberrant physiology replacing the lost function [21].

Microbial Epidemiology Even before the dedicated study of microbiota with molecular, culture-independent techniques, accumulating epidemiologic observations have provided supportive evidence for the presence of microbial dysbiosis in patients with ARDS. First, the most common cause of ARDS is infectious pneumo-

nia, with extra-pulmonary sepsis and aspiration of orogastric secretions comprising the top three etiologies [3]. Consequently, in up to 75% of ARDS cases, microbes are directly implicated in the injurious lung insult. Acute viral pneumonias leading to ARDS, such as influenza and more recently SARS-CoV-2, disrupt the homeostasis of respiratory epithelia and innate immune mechanisms, leading to frequent bacterial or fungal co-infections at the time of ARDS presentation [22, 23]. Regardless of the nature of the initial insult (infectious or not), up to one-third of patients with established ARDS may develop ventilator-associated pneumonia (VAP) further complicating their hospital course [24].

Beyond the injured lungs, the oropharynx of critically ill patients with ARDS has been long shown to be colonized with pathogenic gram-negative rods involved in the development of VAP [25]. De-colonization interventions with antiseptics and antibiotics against oropharyngeal and/or gastrointestinal microbes have consistently shown benefit for VAP prevention in clinical trials [26]. Furthermore, nearly all patients with ARDS receive either microbiology-guided or empiric broad-spectrum antibiotics in the ICU [27], which explains, at least in part, the considerable risk for *Clostridium difficile* infection in mechanically ventilated patients [28]. As a prototypical example of a disrupted microbial community, *Clostridium difficile* colitis predisposes to subsequent systemic infections, given that immediately following hospital discharge for an episode of *Clostridium difficile* colitis, patients are at 70% increased risk for re-hospitalization with sepsis [29].

Taken together, the aforementioned epidemiologic observations have provided the basis for proposing a plausible *microbiome hypothesis* in ARDS pathogenesis [30]. In order to systematically analyze the potential role of microbiota in ARDS, we first need to consider the current ecological theories for the formation of the human microbiome, and especially the models that explain how lung and gut microbial communities are shaped in critically ill patients receiving invasive mechanical ventilation.

Ecological Models for the Lung Microbiome of Critical Illness The most widely cited and accepted ecological theory for the formation of the lung microbiome in health is the *adapted model of island biogeography* [31–34]. According to this model, the high microbial biomass upper respiratory tract (URT) is considered the primary source of microbes for the lower respiratory tract (LRT). The LRT is conceptualized as a microbial island whose members are influenced by the competing rates of microbial immigration from the URT "mainland" (through inhalation, micro-aspiration, or mucosal dispersion) and elimination from the LRT "island" (through mucociliary clearance, cough, and host defense) [31–35]. The net balance of these two opposing forces shapes a highly diverse, but low biomass, lung microbiome in health. The lung microbiome may also be impacted by regional environmental conditions that dictate the reproductive rates of microbes, including nutrient availability, host innate immunity, and oxygen availability. Importantly, such conditions in the healthy, well-aerated lungs are not conducive to bacterial proliferation, explaining the low microbial biomass in the healthy lung [32].

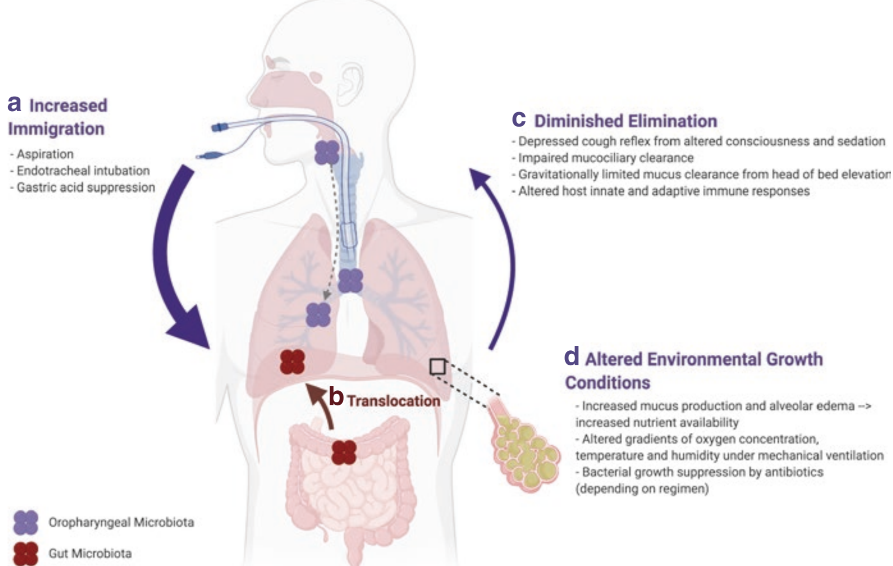

Fig. 11.1 The ecological model for the formation of the lung microbiome in patients with acute lung injury on mechanical ventilation. According to the adapted model of island biogeography, the high biomass upper respiratory tract (URT) is conceptualized as a microbial "mainland" that represents the main source of microbiota for the lower respiratory tract (LRT- microbial "island"). (**a**) Increased immigration of microbes from the URT to the LRT tract occurs due to micro-aspiration of microbes pooling around the endotracheal tube or from proliferating microbes from the stomach. (**b**) Translocation of intestinal microbiota may occur via the venous or lymphatic circulation. (**c**) Decreased microbial elimination can occur due to cough suppression, impaired mucociliary clearance, and altered host immunity. (**d**) Altered environmental growth conditions in the LRT due to alveolar edema rich in nutrients, abnormal oxygen/temperature gradients, etc

Applying the adapted model of island biogeography for the respiratory tract ecology of a critically ill, mechanically ventilated patient with ARDS, it becomes apparent that both pathophysiological mechanisms of disease as well as critical care interventions may profoundly impact the microbial community (Fig. 11.1) [20, 21]. First, patients are exposed to higher rates of microbial immigration along the intubated respiratory tract (Fig. 11.1a). Although the cuffed endotracheal tube is designed to protect the LRT from large volume macro-aspiration, constant micro-aspiration around the tube is inevitable [36], often involving either oropharyngeal colonizers or gastric bacteria, which can proliferate due to prophylactic acid suppression [36, 37]. Another potential source for immigrating bacteria into the lungs is through translocation from the gut, arriving in the lungs via the systemic or the lymphatic circulation (Fig. 11.1b), a biologically plausible phenomenon that has nonetheless been difficult to study and prove in vivo [38, 39].

On the other hand, microbial elimination from the intubated respiratory tract is overall diminished (Fig. 11.1c). Contributing factors include cough suppression due to encephalopathy and sedative medications, impaired secretion clearance through the endotracheal tube, routine head of the bed elevation leading to gravitational

disadvantage for airway clearance, and altered host immunity for microbial killing. The net balance of increased immigration and diminished elimination leads to an absolute increase of microbial load in the lungs of patients with ARDS.

The environmental growth conditions of the alveolar space are also drastically altered in ARDS (Fig. 11.1d), whereby the normally aerated and nutrient-poor alveolar sacs of healthy lungs are depleted of bactericidal surfactant [40, 41] and flooded with protein-rich edema and cellular debris, which represent microbial nutrients. The hallmark heterogeneous distribution of parenchymal injury in ARDS creates regions of aerated lungs exposed to supra-physiologic concentrations of oxygen (up to 100%) vs. completely consolidated, unaerated pockets allowing anaerobic bacterial growth. Similar differences in humidity and temperature gradients can generate selective growth conditions for pre-existing low abundance bacteria or for newly immigrating microbes. The nearly ubiquitous exposure of patients with ARDS to systemic, broad-spectrum antibiotics [27] may lead to global reduction of sensitive bacteria in the lungs, with potential resistant bacteria or fungal overgrowth leading to secondary VAP development [42, 43].

Ecological Models for the Gut Microbiome of Critical Illness As with lung microbiota, the formation of gut microbial communities can be considered as the composite result of the forces of immigration, elimination, and regional growth in the gastro-intestinal tract [20]. However, in contrast to the low biomass lung microbiome in health, the healthy gastro-intestinal tract is abundant with commensal microbes, accounting for the majority of the estimated 39 trillion bacteria in the human body [44]. The large biomass of intestinal bacteria (~0.2 kg in an average size human) exerts key biological functions for the host, including nutrient and hormone metabolism, modulation of immune responses, maintenance of mucosal barrier homeostasis, and protection against invading pathogens [21, 45]. Therefore, gut microbiome disruption in critical illness needs to consider not only changes in absolute biomass but also changes in community architecture and associated host metabolic functions. Specifically for patients with ARDS, we consider the following mechanisms that can impact gut microbiota:

(a) Systemic hypoxemia and hypoperfusion can impair intestinal mucosal barrier and immunity function.
(b) Widespread use of antibiotics can indiscriminately ablate indigenous microbiota and allow proliferation and expansion of resistant pathobionts.
(c) Delayed or lack of enteral feeding can deplete commensal microbes from luminal nutrients.
(d) Sedation or illness-related ileus can lead to regional bacterial overgrowth [20, 21].

The end-result of an altered, pathogen-enriched gut microbiome has been proposed as a driver of critical illness [46, 47], leading to systemic pathogen invasion (*microbial translocation*) and mediating distal end-organ damage by release of inflammatory mediators (*gut-lymph hypothesis*) [38, 46].

Apart from the ecological models that predict the formation of the lung and gut microbial communities, we also need to consider the pathogenetic causal models that

would explain the involvement of microbiota in ARDS. Such causal modeling is necessary in order to propose testable hypotheses and design appropriate studies. From a broader perspective, inclusion of the microbiome in ARDS causal models will allow us to expand from the traditional model predicting unidirectional development of ARDS following exposure to a direct or indirect injurious risk factor (Fig. 11.2 left panel). We thus need to take into account the potential interactions between exposures, microbiota (lung or gut), and the injured lungs of ARDS (Fig. 11.2 right panel) [30]. Defining the directionality of these host-microbiome interactions will help then clarify the role of microbiota. First, we consider an exposure that directly alters the lung microbiome (e.g., viral or bacterial pneumonia), which in turn leads to lung inflammation and injury (Fig. 11.2, arrow a). Conversely, established lung injury by microbiota-unrelated factors (e.g., inhalational injury or trauma) can alter the respiratory ecosystem, thereby influencing the lung microbiome composition (Fig. 11.2, arrow b). In such a scenario, the perturbed lung microbiome would be a consequence of lung injury, i.e., a non-causal epiphenomenon. In perhaps a more plausible and complex scenario, following the establishment of lung dysbiosis and injury, these two factors can influence and perpetuate each other in a positive feedback loop (Fig. 11.2, arrow c). Such bidirectional host-microbiome interactions may explain the persistent

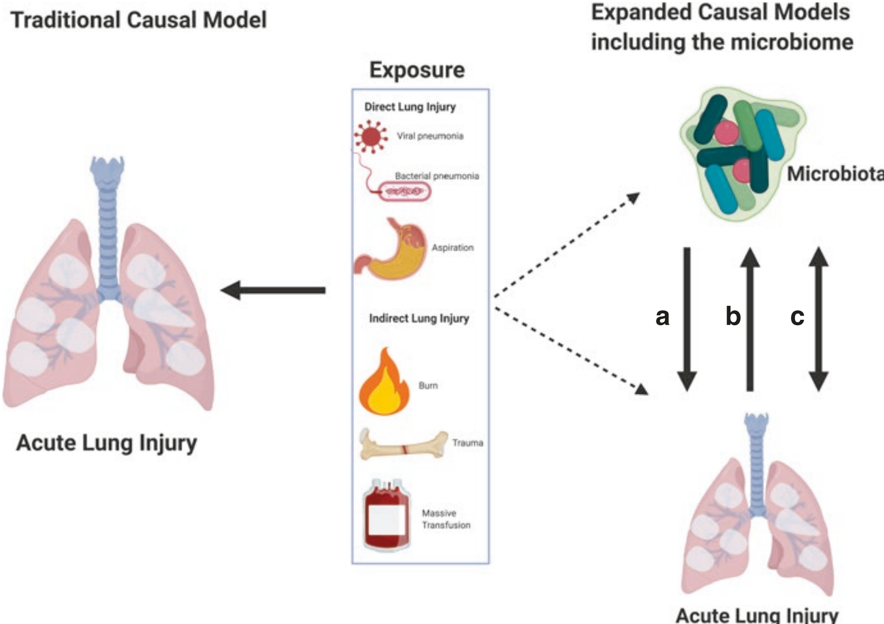

Fig. 11.2 Traditional causal model (left) and expanded causal models (right) for the potential role of microbiota in acute lung injury (ALI). The traditional model considers a unidirectional development of ALI following exposure to a risk factor (direct or indirect). The expanded model considers the impact of risk factors both on microbiota and the injured lungs, and the potential interactions between them (model adapted from [30]). Although this expanded model was proposed for lung microbiota, it can also be applied for the role of gut microbiota in ARDS

lung injury observed in many patients with ARDS even when the initial trigger has resolved. To comprehensively address these three core hypotheses, complementary study designs are needed, including longitudinal human studies to examine temporal evolution of communities with appropriate modeling of exposures and confounders, as well as carefully conducted animal model experiments [48].

Sampling Lung and Gut Microbiota in ARDS

Microbiota sampling strategies have been extensively reviewed in Chap. 1. However, some special practical considerations for sampling lung and gut microbiota from critically ill patients are necessary, given the associated procedural risks and sample availability issues.

Lung Microbiota Sampling Bronchoscopic (invasive) sampling is the reference standard methodology for microbiologic work-up in defining the specific etiology of a microbial pneumonia causing ARDS or when concerned for a VAP [24, 42]. By obtaining distal bronchial and alveolar space lavage fluid, bronchoscopic alveolar lavage (BAL) can capture up to 5% of the total alveolar surface [34] and bypasses potentially contaminating URT secretions when performed through the endotracheal tube. Bronchoscopic protective specimen brushes may offer even more details for airway resident microbiota, yet they are not routinely utilized in the care of patients with ARDS. Given the ability to capture large areas of the bronchial tree and alveolar space, BAL samples are generally considered as the reference standards for lung microbiome studies. Nonetheless, bronchoscopic sampling in the critically ill hypoxemic patient with ARDS is not without risks. The transient break of the closed ventilatory circuit for the insertion of the bronchoscope and the partial obstruction of the endotracheal tube cross-sectional area can lead to alveolar de-recruitment and drop in minute ventilation, further compromising gas exchange [49]. The instillation of sterile saline, even if in small amounts (<200 ml), can worsen regional lung edema and hypoxemia in patients with marginal oxygenation. Furthermore, concerns for procedural aerosolization and healthcare personnel exposure to infectious agents (especially SARS-CoV-2) have limited the wide use of BAL sampling for clinical care and research [50]. For these reasons, non-invasive sampling options for the LRT microbiome have been gaining popularity due their wider availability, favorable safety profile, and low cost. Non-bronchoscopic BAL (“mini” or “blind” BAL), performed with a telescoping catheter advanced blindly to a distal bronchus without direct visualization, and the even less invasive endotracheal aspirate (ETA) conducted with simple suctioning of distal tracheal secretions with an in-line catheter available for routine mucus clearance [48] have shown similar outcomes for VAP diagnosis and antimicrobial therapy guidance compared to bronchoscopic sampling in clinical trials [51]. Such non-invasive samples are currently the recommended first-line approach for clinical microbiologic studies in mechanically ventilated patients [52, 53]. Limited comparative data from microbi-

ome studies are available, but similar metagenomic sequencing profiles were obtained in a study of mini-BAL vs. ETA sampling in mechanically ventilated patients with bacterial pneumonia [54].

ARDS is characterized by a hallmark spatial heterogeneity in the prototypical patient ventilated in a supine position. With a gravitational (ventrodorsal) gradient of lung injury and deaeration, the corresponding radiographic heterogeneity is reflected by dorsal atelectasis, mid-zone ground glass opacities and airspace consolidations, and then spared parenchyma in non-dependent, ventral position ("baby lung") [55]. Although invasive sampling techniques with bronchoscopy can be empirically directed toward radiographically abnormal areas to theoretically maximize yield of diagnostic sampling for conventional microbiologic testing, there is paucity of data for any differences in lung microbiota between different areas of heterogeneous lung injury in ARDS. Data from healthy subjects suggest limited regional heterogeneity of microbiota in the normal lung [33]. In contrast, spatial heterogeneity of lung microbiota has been shown in chronic lung disease, including chronic obstructive pulmonary disease [56] or cystic fibrosis, in which phylogenetic and phenotypic differences of colonizing pathogens have been demonstrated between mildly and severely diseased lung regions [57]. In idiopathic pulmonary fibrosis (IPF), a progressive lung disease characterized by an apicobasal gradient of injury and fibrosis, no significant differences in microbiome profiles were found in a study of lung explants that examined apical and basal parenchymal tissue specimens [58]. Nonetheless, higher microbial load was found in airway tissue vs. parenchymal tissue samples, highlighting the possibility of an airway resident microbiome in a chronic lung disease with advanced anatomical destruction of the airspaces by honeycombing [58–60]. Whether sampling more proximal airways via ETA samples vs. consolidated or collapsed airspaces of dorsal lung regions in ARDS would offer different representations of the lung microbiome in ARDS remains to be defined.

Gut Microbiota Sampling For most epidemiological studies of the gut microbiome, stool samples have been utilized rather than invasive endoscopic biopsies for mucosal associated microbiota. Stool samples are noninvasive, inexpensive, and readily available and are widely considered to represent the distal gastrointestinal tract microbiota. However, obtaining stool samples from critically ill patients is not straightforward. Early in the ICU course, stool samples are often not available, either due to constipation or ileus [4] or due to delays in provision of enteral nutrition [5]. Furthermore, standard decontamination practices [6] often result in stool disposal before samples are collected, thus making stool-based studies impractical. Rectal swabs have been proposed as a minimally invasive alternative method of stool sampling, as they can be performed on-demand and are routinely used to screen for colonization with vancomycin-resistant *Enterococcus* in clinical practice. Despite the potential practical advantages, caution is needed when rectal swabs are used for gut microbiota profiling in the ICU. Rectal swabs may offer discordant representations of gut microbiota compared to stool samples in terms of diversity

and composition, especially when performed early in the course of critical illness [61] or when swabs are not visibly soiled by stool [62].

Respiratory Tract Microbiota in Patients with ARDS

A growing body of literature has examined respiratory microbiota in critically ill patients with or at-risk for ARDS. In most studies, secondary analyses of pre-existing ICU cohorts were conducted, and given the practical limitations of obtaining repeated LRT samples in patients with ARDS, mostly noninvasive cross-sectional samples have been utilized. In our review of the best available evidence, we consider studies enrolling critically ill subjects receiving invasive mechanical ventilation, with established ARDS or at risk for its development due to the temporal presence of lung injury risk factors. Our review is not exhaustive for all available studies in the field, which have been summarized elsewhere [20, 21]. Rather, we analyze important study design aspects of the highest impact articles to date and provide a graphical summary of study findings across the URT and LRT (Fig. 11.3). Importantly, the lung microbiota profiles provided from each study must be interpreted in the context of the available knowledge about the healthy lung microbiome, which has been characterized by the following four main features:

1. *Low biomass*: BAL fluid from healthy subjects analyzed by 16S rRNA gene quantitative polymerase chain reaction (qPCR) has much lower bacterial burden (estimated bacterial density ~ 10^{2-3} bacteria/ml of BAL fluid) [63–67] compared to oral wash fluid (density estimates ~10^{5-6} bacteria/ml of oral wash) [63, 64, 67].
2. *High alpha diversity*: Communities from healthy subjects are generally characterized by higher alpha diversity indices compared to diseased states [68].
3. *Oral similarity*: LRT communities exhibit compositional similarity to corresponding oral (and not nasal) communities in adults [64, 67–70].
4. *Limited regional heterogeneity*: Bronchoscopic sampling of multiple lung sites has shown greater between- than within-subjects differences, i.e., a given individual's right middle lobe community more closely resembles the same individual's left upper lobe than another individual's right middle lobe [33]. Therefore, a single sample from each patient is considered to be representative of his or her lung microbiome.

Studies Exclusively in Patients with ARDS or Examining ARDS as an Outcome

In the largest cohort with longitudinal sampling prior the development of ARDS, Panzer et al. enrolled 74 mechanically ventilated patients with blunt trauma and collected paired baseline (0 hr) and follow-up (48 hrs) ETA and blood samples in an

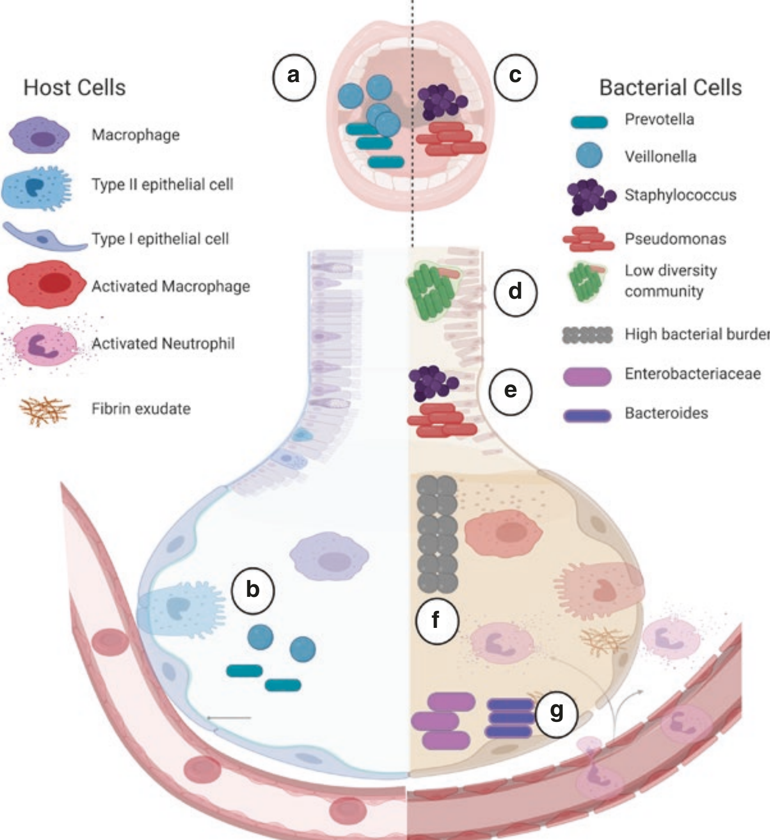

Fig. 11.3 Visual summary of findings from human studies in acute lung injury for the microbial community of profiles of the upper and lower respiratory tract. The upper panel depicts the upper airway and the lower panel the lower respiratory tract (LRT) and the alveolar-capillary interface. The left side of the panel depicts homeostatic conditions in health, whereas the right side of the panel depicts the pathophysiologic derangements of acute lung injury (ALI) resulting in the clinical manifestation of acute respiratory distress syndrome (ARDS). Important human (host) cells are highlighted. Bacterial cells are shown in sizes out of proportion relative to the human cells to allow visualization. (**a**) The upper respiratory tract (URT) comprises a high biomass, high diversity community of commensal microbiota (mainly *Prevotella* and *Veillonella* species) [68]. (**b**) The LRT comprises a low biomass, high diversity community of oral-origin bacteria (e.g., *Prevotella* and *Veillonella* species) [68, 70, 71]. (**c**) In mechanically ventilated patients with ARDS, up to 45% of patients have displacement of the typical oral-microbiota and altered composition of communities with enrichment for typical respiratory pathogens (e.g., *Staphylococcus* or *Pseudomonas* taxa) [18]. (**d**) By endotracheal aspirate (ETA) sampling of LRT secretions, low alpha diversity communities have been associated with worse clinical outcomes [18, 72]. (**e**) High relative abundance of pathogenic taxa (e.g., *Staphylococcus* or *Pseudomonas*) strongly correlates with corresponding pathogenic species identification by microbiologic cultures [73–75]. A pathogen-enriched cluster in ETA samples has been independently associated with hyperinflammatory host-responses and worse clinical outcome [18]. (**f**) High bacterial burden by 16S rRNA gene quantitative PCR in bronchoscopic alveolar lavage samples has been independently associated with worse clinical outcome [19, 75]. (**g**) Patients with ARDS are often enriched with gut-origin bacteria (*Enterobacteriaceae* and *Bacteroides* taxa) [19, 76, 77]

effort to understand the role of lung microbiota in the development of ARDS following severe trauma [76]. They also examined for potential effect modification by smoking exposure (quantified via plasma cotinine levels) on the association between lung microbiota and indirect lung injury from severe trauma. ARDS development was significantly associated with relative enrichment of ETA communities for *Enterobacteriaceae*, a gut-origin taxon, as well as two taxa of oral origin (*Prevotella* and *Fusobacterium* Operational Taxonomic Units – OTUs) at 48 hrs based on 16S rRNA gene sequencing, both of which were enriched in smokers at baseline. Notably, baseline microbiota were not predictive of ARDS development. Instead, the evolution of communities by 48 hrs was significantly associated with ARDS, providing temporal evidence for a potential causal role for community enrichment with putative pathogens preceding lung injury. In further support of the role of lung microbiota, both baseline and 48 hr. taxonomic composition were associated with plasma biomarkers of epithelial (vascular endothelial growth factor, receptor for advanced glycation end-products) and endothelial (intercellular adhesion molecule-1, angiopoeitin-2) injury and inflammation (interleukin [IL]-6, IL-8 and pentraxin-3), which carry independent prognostic information in ARDS [15, 78].

In a secondary analysis of BAL samples from patients with ARDS enrolled in a previous clinical trial, Dickson et al. showed that lung communities were enriched with an uncultured, anaerobic member of the *Bacteroides* genus [77] in 33% of BAL samples from patients with ARDS, as opposed to only 3% of healthy subjects, confirming that the organism is not a normal community member of the healthy lung microbiome. The lung enrichment with gut-specific bacteria was not significantly associated with the predisposing factor for ARDS or severity of illness but correlated significantly with systemic inflammation measured by serum tumor necrosis factor alpha (TNF-α) concentration. These findings underline the plausibility of a gut-lung translocation mechanism in critically ill patients that may explain the appearance of gut origin bacteria in the alveolar space.

Kyo et al. performed a case-control study of 40 patients with ARDS compared to seven mechanically ventilated controls, by profiling BAL microbiota with 16S sequencing and measuring plasma and BAL inflammatory cytokines [75]. A quarter of ARDS patients were diagnosed with bacterial pneumonia, with cultured pathogens corresponding to dominant taxa by sequencing. However, bacterial pneumonia diagnosis was not associated with higher culture-independent bacterial load in BAL samples by 16S qPCR, whereas ARDS patients appeared to have higher bacterial load compared to controls. Among ARDS non-survivors (n = 16), higher plasma IL-6 levels correlated with higher bacterial load by qPCR, as well as higher relative abundance for *Staphylococcus*, *Streptococcus*, and *Enterobacteriaceae* taxa.

Schmitt et al. conducted a case-control study of 15 patients with ARDS from indirect lung injury (major abdominal surgery for sepsis) compared to 15 mechanically ventilated controls post esophageal surgery [79]. With 16S sequencing of BAL samples, ARDS patients had significantly lower alpha diversity compared to controls (lower richness, higher dominance), and this low alpha diversity at baseline predicted worse clinical outcomes, including length of ICU stay and duration of mechanical ventilation. Patients with ARDS had distinct taxonomic composition

from controls, with decrease in typical oral taxa abundance (mainly *Prevotella* and *Veillonella* taxa).

Finally, Walsh et al. conducted a case-control study of patients who developed ARDS compared to those who did not following burn and inhalational injury [80]. All patients underwent diagnostic bronchoscopy for inhalational injury confirmation and were subjected to therapeutic washes. By 16S sequencing of bronchial washings, patients with hypoxemia (PaO2/FiO2 ratio < 300) had differences only in low abundance taxa compared to patients with PaO2/FiO2 ratio > 300, with higher relative abundance of *Prevotella melaninogenica* in hypoxemic patients being the most robust finding in this study.

Studies in Mechanically Ventilated Patients with or at-Risk for ARDS

Lamarche et al. prospectively followed 34 mechanically ventilated patients at-risk for ARDS, in a cohort enriched for pneumonia and sepsis, nested within a randomized clinical trial of a *Lactobacillus*-based probiotic [72]. The investigators collected 29 ETA and 26 gastric aspirates. Notable findings included considerable compositional overlap between ETA and gastric aspirate samples, as well as the loss of specific bacterial taxa from ETA samples compared to healthy controls, including lower abundance for *Neisseria*, *Veillonella*, *Streptococcus*, *Staphylococcus*, and *Corynebacterium* taxa. Lower ETA alpha diversity correlated with severity of illness by APACHE-II scores and was associated with increased risk of death. In contrast, alpha diversity was not associated with diagnosis of pneumonia or concomitant antimicrobial exposure. Overall, in this cohort, pathogen expansion appeared to be a patient-specific process, whereas loss of commensal bacteria from body sites was a more generalized phenomenon.

Kelly et al. enrolled a cohort of 15 mechanically ventilated patients and collected matched oropharyngeal swab and ETA samples within 24 hrs of intubation and then serially every 72 hrs thereafter [74]. In contrast to healthy controls, URT and LRT samples form critically ill subjects demonstrated decreased alpha diversity by 16S sequencing, which declined further over time while on ventilatory support. Many communities were dominated by a single taxon, which correlated with culture data in patients with microbiologically confirmed pneumonia.

Dickson et al. conducted a secondary analysis of mini-BAL specimens collected within 24 hrs of ICU admission from 91 critically ill patients (19% with ARDS) that had been enrolled in the Molecular Diagnosis and Risk Stratification of Sepsis project [19]. By 16S qPCR, patients with ARDS had significantly higher bacterial load compared to patients without ARDS ($p = 0.01$), and by 16S sequencing, the *Enterobacteriaceae* taxonomic group was the most discriminating feature between ARDS and non-ARDS specimens. Strikingly, the most prominent *Enterobacteriaceae* OTU was 96% aligned with the *Enterobacteriaceae* OTU associated with ARDS in

the study by Panzer et al. [76]. Of the baseline microbiome profile features (bacterial load, diversity, and community composition), higher bacterial burden and community composition enriched for gut-associated taxa were significantly associated with worse ICU outcomes.

Our group has recently published the results of the largest cohort study of respiratory microbiota in mechanically ventilated patients to date [18]. We enrolled 301 critically ill patients with acute respiratory failure (24% with ARDS) and performed matched sampling of the URT and LRT with oropharyngeal swabs and ETA samples, respectively. By 16S sequencing, critically ill patients had markedly different profiles than corresponding healthy controls and exhibited substantial heterogeneity in alpha- and beta-diversity. To agnostically uncover informative subsets of microbial profiles, we performed unsupervised clustering with Dirichlet Multinomial Models (DMM), which revealed a cluster with low alpha-diversity and enrichment for pathogens (e.g., high *Staphylococcus* or *Pseudomonadaceae* relative abundance) in 35% of ETA samples, associated with a hyperinflammatory subphenotype of host-responses, worse 30-day survival and longer time-to-liberation from mechanical ventilation (all adjusted $p < 0.05$), compared to patients with higher alpha-diversity and relative abundance of typical oral microbiota. A similar pathogen-enriched cluster emerged in the oral communities, also significantly associated with worse clinical outcomes. Patients with evidence of dysbiosis (low alpha diversity and low relative abundance of "protective" oral-origin commensal bacteria) in both URT and LRT communities (17%, combined dysbiosis) had significantly worse 30-day survival and time-to-liberation compared to patients without dysbiosis (55%, adjusted $p < 0.05$).

The available evidence from the study of respiratory microbiota in ARDS and acute respiratory failure offers the following take-home messages:

- LRT communities in patients who develop ARDS are often enriched with gut-origin bacteria (especially *Enterobacteriaceae* taxa) [19, 76, 77], and such enrichment may precede the onset of ARDS [76].
- Lung microbial dysbiosis is associated with systemic host inflammatory responses [18, 75–77].
- Low community diversity in ETA samples [18, 72] and high bacterial burden in BAL samples are predictive of adverse clinical outcomes [19, 75].
- Paired analyses of URT and LRT samples have revealed predictive patterns of dysbiosis along the respiratory tract [18, 74].

The Gut Microbiome in Patients with ARDS

Limited evidence from human studies is available for the role of gut microbiota in ARDS. The gut microbiome has been more thoroughly studied in patients with sepsis in the ICU, in accordance with the gut-origin theory of sepsis [21, 46, 47].

Several studies have also examined the gut microbiome as a potential pathogen reservoir for subsequent hospital-acquired infections [81–84]. No study of gut microbiota has specifically focused on patients with ARDS, albeit a number of studies have enrolled patients receiving invasive mechanical ventilation, which we review below. Interpretation of the gut microbiome profiles identified in ICU studies has to consider the extensive knowledge about the healthy gut microbiome, established as a high biomass and highly diverse community, with high abundance of obligate anaerobes from the phyla Firmicutes and Bacteroidetes [85].

McDonald et al. enrolled 115 mechanically ventilated patients from four ICUs and collected stool samples at two time points for 16S sequencing, within 48 hrs of admission and at ICU discharge or day 10 [86]. Alpha diversity at discharge was lower than at admission. When compared to healthy control samples from the American Gut Project, stool samples from ICU patients had lower relative abundance of taxa from the Firmicutes and Bacteroidetes phyla and increased abundance of Proteobacteria. Organisms previously thought to confer anti-inflammatory benefits, such as *Faecalibacterium*, were largely depleted, whereas "inflammatory" taxa, as, for example, members of the *Enterobacteriaceae* family, were increased in abundance.

Burmeister et al. collected rectal swabs at the time of admission from 67 patients who suffered severe trauma and were admitted to a level I trauma center [87]. By 16S sequencing, patients who developed ARDS and non-survivors had significantly different taxonomic composition compared to their counterparts. Non-survivors ($n = 8$) had increased abundance of specific taxa, including *Eubacterium biforme*, *Ruminococcus flavefaciens*, *Akkermansia muciniphila*, and *Oxalobacter formigenes*.

Shimizu et al. conducted a randomized clinical trial in mechanically ventilated patients with sepsis by administering a synbiotic cocktail (including *Bifidobacterium breve, Lactobacillus casei,* and galacto-oligosaccharides) upon initiation of enteral nutrition vs. standard of care [88]. Patients treated with synbiotics had much lower incidence of VAP compared to the control group (14.3% vs 48.6%, $p < 0.05$). Sequencing analysis of fecal bacteria revealed that probiotics *Bifidobacterium* and *Lactobacillus* had successfully engrafted, with higher abundance than in the control group, as well as higher concentration of the beneficial short-chain fatty acid (SCFA) acetate. Thus, synbiotic modulation of gut microbiota may protect patients from the complication of VAP while on mechanical ventilation.

In a prospective cohort of mechanically ventilated patients at risk for ARDS following cardiothoracic surgery, Aardema et al. collected fecal samples before surgery, post-operatively in the ICU and then at outpatient follow-up [89]. 16S sequencing revealed reduction of alpha diversity during ICU admission, which was further associated with longer hospital stay. Strictly anaerobic bacteria that produce SCFA were depleted, with a corresponding increase in pathobionts. For patients with available post-discharge samples, their gut microbiota profiles had reversed to pre-admission status, indicating the transient nature of microbiota shift during brief critical illness.

Despite the limited and mainly circumstantial evidence available for gut microbiota in ARDS from human studies, the following take-home messages can be deducted:

- Alpha diversity is decreased in the gut communities of critically ill mechanically ventilated patients.
- Critical illness is associated with loss of Firmicutes and Bacteroidetes taxa with converse increase in Proteobacteria in the gut.
- Increase in potential pathogens in gut communities is associated with future risk of infection.
- Gut microbiota can be modulated with synbiotics for the prevention of VAP.

Animal Model Studies for the Role of Microbiota in Acute Lung Injury

Animal modeling is a powerful tool for mechanistic interrogation of microbiota in respiratory disease [77, 90–92] allowing for control of microbial exposure in ways that are not possible for human studies. The animal microbiome can be manipulated with the use of germ-free animals, microbiota depletion with intensive antibiotic treatments, introduction of specific bacteria (e.g., gnotobiotic animals) [93], or even complete microbial replacement (e.g., with fecal transplant or oral gavage) [48]. Combination of these tools can help dissect causality in microbiome-outcome associations and allow for testing of therapeutic interventions targeting the microbiome. Murine modeling has been the predominant format in the microbiome literature, and of note, LRT sampling in mice is most reproducibly achieved by utilizing whole lung homogenate instead of BAL [94, 95].

Despite the strengths of animal models, there are important caveats in the study of the ALI microbiome that need to be taken into account in interpreting published studies:

(a) *Difficulties reproducing the pathological substrate of ALI*: the pathological features of human ARDS in the initial exudative phase (~7 days) involve DAD, influx of neutrophils, and formation of microthrombi [8]. Although many animal models of direct and indirect lung injury have been developed, there is no single ALI model that truly reproduces all features of human ARDS [96, 97]. Therefore, it is important to note that each ALI model may be only partially testing the underlying pathobiology of human ARDS, and the model of choice depends on the specific question to be addressed.

(b) *Difficulties modeling the intensive care provided in ARDS*: ARDS is a multiple-hit process with resultant multi-organ dysfunction, necessitating a bundle of life-saving interventions including respiratory and hemodynamic support. The complexity makes it difficult to disentangle the interactions between the lung injury etiology and the concurrently administered interventions, which themselves can further exacerbate ALI (e.g., with ventilator-associated lung injury or hyperoxia) [8]. Most animal models induce ALI by delivering a single noxious agent in genetically similar animals and do not capture the complex network of

interactions occurring from the provision of critical care in patients with ARDS [98].

(c) *Confounders on animal microbiota*: Despite genetic similarity and control of exposures in a given animal experiment, such studies are not free from confounding [48]. Accumulating empirical evidence has shown the influences of cage, bedding, temperature, and co-housing on murine microbiota [94]. Mice are coprophagic and when co-housed, they demonstrate a time-dependent convergence of their lung microbiota. Animal sex may also influence lung and gut microbiota, but this is rarely considered in experiments owing to cohousing concerns. Guidance for careful control of confounders when studying the microbiome in animal models has been proposed [99].

(d) *Murine* vs. *large animal models*: although mice are the most commonly used animals in the ALI literature and the murine lung microbiome has been well catalogued, there are important anatomical and ecological differences between the murine and human respiratory tracts [48]. Therefore, inferences drawn on the clinical relevance of murine microbiome studies in ALI need to be cautious. Larger animals such as pigs, sheep, and rabbits are better suited for more complex physiologic measurements in ALI but are much more expensive, and their microbiota have not been as well studied.

With the aforementioned caveats of animal modeling in mind, the expanding experimental literature has offered novel insights for the role of microbiota in ALI. In Table 11.1, we provide a detailed summary of the most relevant aspects of study design and findings for key animal studies published to date. The take-home messages from this body of literature include:

– There is an interplay between the host's environment and lung microbiota in the pathogenesis of ALI. Recent animal experiments demonstrated that exposure to hyperoxia reduced the abundance of anaerobic taxa and increased abundance of oxygen-tolerant taxa (e.g., *Staphylococcus*) but failed to induce ALI in germ-free mice [100].
– Microbiota can mediate ALI from additional iatrogenic causes beyond hyperoxia [100], such as from high tidal volume ventilation or blood product transfusion [101, 102].
– The role of the microbiome is heavily context-dependent: microbiota can harm [102] or protect [101] from ALI depending on the mechanism of injury and experimental setting.
– Microbiota can translocate from the gut to the lungs in the case of an indirect ALI insult [77] but also in the opposite direction in the case of a direct ALI insult [103].
– The lung microbiome is altered following ALI, and the altered ALI microbiome can further intensify lung inflammatory injury [91].

Table 11.1 Study design characteristics and main findings for animal models studies examining lung or gut microbiota in acute lung injury

Article	Study design	Summary of study findings
Ashley et al. 2020 [100]	**Animal model:** C57BL/6 mice **ALI inciting factor:** hyperoxia **Microbiota studied:** lung and gut **Microbiota manipulation:** germ-free animals, enteral and intraperitoneal antibiotics **ALI outcome:** Alveolar protein, neutrophilia, IgM concentration and lung histology	Mice exposed to hyperoxia developed altered lung and gut microbiota, with decline of anaerobes and enrichment with oxygen-tolerant taxa (e.g. *Staphylococcus*) Hyperoxia-induced lung dysbiosis preceded peak lung injury In hyperoxia exposed mice, variation in lung inflammation correlated with variation in lung microbial communities Germ-free mice were protected from oxygen induced lung injury Intraperitoneal ceftriaxone exacerbated the degree of hyperoxia-induced lung injury Bronchoalveolar lavage fluid from hyperoxia exposed mice promoted the growth of *S.aureus* in ex vivo culture
Poroyko et al. 2015 [91]	**Animal model:** C57BL/6 mice **ALI inciting factor:** intratracheal LPS **Microbiota studied:** lung **Microbiota manipulation:** BAL microbiota transfer **ALI outcome:** Alveolar protein, cell counts, neutrophilia, Evans blue dye extravasation, and lung histology	Bacterial load increased 5-fold in BAL fluid of LPS-treated mice The increased bacterial load was accounted for by a bloom of indigenous Proteobacteria capable of metabolizing the nutrients of the BAL fluid By culture of BAL fluid, two distinct colonies were identified for *Stenotrophomonas maltophilia* and *Ochrobactrum anthropi* Intratracheal administration of BAL microbiota from LPS-treated mice did not cause ARDS in naïve mice, but intensified IL-6-induced lung inflammation in mice treated with IL-6, suggesting that the altered microbiome can act as an effect modifier in ARDS following an initial insult
Wienhold et al. 2018 [101]	**Animal model:** C57BL/6 mice **ALI inciting factor:** high tidal volume ventilation **Microbiota studied:** gut **Microbiota manipulation:** enteral antibiotics **ALI outcome:** Alveolar cell counts, neutrophilia, lung permeability, ventilatory mechanics, and lung histology	Disruption of gut microbiota by enteral antibiotic therapy prior to mechanical ventilation increased the susceptibility to ventilator-induced lung injury, as shown by increased pulmonary permeability, increased oxygenation index, decreased pulmonary compliance, enhanced macroscopic lung injury and increase cytokine levels in lung homogenates Antibiotic treated mice subjected to high tidal volume ventilation had elevated inflammatory responses, but non-ventilated control mice with depleted microbiota from antibiotics did not have elevated inflammatory responses

(continued)

Table 11.1 (continued)

Article	Study design	Summary of study findings
Mohammed et al. 2020 [129]	**Animal model:** C3H/Hej mice **ALI inciting factor:** Staphylococcal Enterotoxin B (SEB) intranasally/intraperitoneally **Microbiota studied:** gut **Microbiota manipulation:** fecal microbiota transplant, tetrahydrocannabinol (THC) **ALI outcome:** lung histology	Staphylococcal Enterotoxin B (SEB)-induced ALI is associated with loss of protective microbes in the gut and lung communities, such as *Ruminococcus gnavus*, and increase in abundance for pathogenic microbiota such as *Akkermansia mucuniphila* THC significantly increased the abundance of beneficial *Ruminococcus gnavus*, but decreased pathogenic microbiota, such as *Akkermansia muciniphila* THC treatment also led to increase in short-chain fatty acids (SCFA), of which propionic acid was found to inhibit the inflammatory response FMT from SEB + THC treated mice lead to rescue of the fatal ALI phenotype of SEB-challenged mice
Kapur et al. 2018 [102]	**Animal model:** C57BL/6 mice **ALI inciting factor:** Transfusion related acute lung injury (TRALI) monoclonal antibodies **Microbiota studied:** gut **Microbiota manipulation:** fecal microbiota transplant, antibiotics, specific pathogen free mice housing **ALI outcome:** alveolar neutrophilia, wet/dry lung ratios, lung histology	Barrier-free (BF) mice were susceptible to antibody-mediated murine TRALI, whereas specific-pathogen free (SPF) and antibiotic-treated BF mice were protected from TRALI TRALI susceptibility could be restored in SPF mice by pretreating the mice with a low dose of LPS or via BF fecal transfer This study identified a previously unestablished link between the gut microbiome and the onset of antibody-mediated murine TRALI
Dickson et al. 2016 [77]	**Animal model:** C57BL/6 mice **ALI inciting factor:** cecal ligation and puncture (CLP), intratracheal LPS **Microbiota studied:** lung and gut **Microbiota manipulation:** antibiotics **ALI outcome:** alveolar inflammation	Following septic insult with CLP, lung communities were dominated by an uncultured bacterium, OTU008 (*Bacteroidales* sp.) that normalized after 2 weeks This bacterium did not dominate the bacterial communities detected in simultaneously collected tongue specimens but was the most abundant community member in all lower gastrointestinal sites of mice before injury *Enterococcus faecalis* (the second most abundant species) detected in the lungs of mice 5 days after sepsis) was isolated via aerobic culture from the lungs of all post-sepsis mice 5 days after injury Mice exposed to direct lung injury via intratracheal LPS instillation did not have Enterococcus faecalis isolated from their lungs This study demonstrates that that the lung microbiome is enriched with gut bacteria in experimental sepsis and indirect lung injury

Schuijt et al. 2016 [130]	**Animal model:** C57BL/6 mice **ALI inciting factor:** intranasal administration of *S. pneumoniae* **Microbiota studied:** gut **Microbiota manipulation:** antibiotics **ALI outcome:** alveolar inflammation, lung histology	Mice with depleted microbiota by broad spectrum antibiotics had higher mortality and increased bacterial load in their lungs 6hrs post pneumococcal challenge At 6h post-infection, gut microbiota depleted mice demonstrated early and significantly more inflammation compared to control mice. Histopathology demonstrated enhanced interstitial inflammation, endothelialitis, and edema FMT to gut microbiota-depleted mice restores pulmonary bacterial clearance. Gut microbiota depleted mice have reduced blood and lung TNF-α levels, cytokine, and CXCL1 levels that are restored with FMT
Prakash et al. 2015 [131]	**Animal model:** C3H mice **ALI inciting factor:** left pulmonary artery occlusion and reperfusion **Microbiota studied:** gut **Microbiota manipulation:** antibiotics **ALI outcome:** lung histology	Mice with depleted gut microbiota have attenuated inflammation identified by histology and reduced inflammatory plasma IL-6, CXCL2/MIP2ζ, and CCL2/MCP1 Mice treated with intestinally localized antibiotics have an attenuated systemic and localized inflammatory response to ischemic lung injury induced by transient pulmonary artery occlusion
Sze et al. 2014 [103]	**Animal model:** C57BL/6 mice **ALI inciting factor:** intratracheal LPS **Microbiota studied:** lung, gut, blood **Microbiota manipulation:** antibiotics **ALI outcome:** alveolar cell counts	Following endotracheal LPS instillation, total bacterial count increased in the cecum and the blood, while antibiotics abrogated the increase in the blood and not in the cecum Increase of total bacteria correlated with the taxon *Phyllobacteriaceae OTU 40* Similarity of communities and BAL and blood post-LPS instillation suggested possibility of translocation of bacteria from the lungs into the bloodstream

Putting Everything Together: The Microbiome Matters in ARDS

The observational findings from human studies and the experimental data from murine ALI models provide clear-cut evidence that the microbiome is relevant in ARDS. Mainly focused on the respiratory tract, human studies have revealed that the LRT microbiota are reproducibly altered in ARDS or in the presence of inflammatory risk factors, with higher bacterial burden, lower alpha diversity, loss of commensal oral bacteria, and enrichment for putative pathogens. Dysbiotic communities are associated with systemic host inflammation and predict worse patient-centered clinical outcomes [18, 19, 75, 77]. Although human studies cannot establish causality or direction of effects, the reproducible signal, biological plausibility, and temporality of observed associations (i.e., baseline microbiota predicting ICU outcomes) are strongly supportive of an important and pathogenetic role of microbiota in ARDS. The murine studies have confirmed the causal role of microbiota in specific contexts of ALI generation, evolution, and response to therapy. Therefore, the totality of evidence suggests that the microbiome is not an innocent bystander (Fig. 11.2b hypothesis) but can act both as an instigator (Fig. 11.2a) as well as a perpetuator of ALI (Fig. 11.2c).

The precise mechanisms by which the lung microbiome influences ARDS pathogenesis remain unclear. Given the heterogeneous nature of ARDS etiology, severity, response to treatment, and outcomes, lung or gut microbiota may be involved by several mechanisms and in different stages of ARDS evolution. The temporal relationship of dysbiosis preceding ALI in animal models of direct lung injury insults [e.g., hyperoxia [100] or bleomycin [92]] support a direct causal role of perturbed lung microbiota in precipitation of ALI. Possible mechanisms include:

- Stimulation of innate immune cells by microbiota through pattern recognition receptor (PRR) signaling [104]
- Inflammatory damage from chemokine-driven neutrophilic influx in the alveolar space [e.g., by CCL2/CCL7 [105, 106]]
- Direct damage or invasion of pneumocytes by viruses or intracellular bacteria [107, 108]
- Excretion of molecules and metabolites that can propagate inflammation [109] or induce apoptosis in lung epithelial cells [110]
- Depletion of beneficial metabolites (e.g., short-chain fatty acids) produced by gut commensal bacteria that influence lung immune defenses [111–113]
- Translocation of microbes (or their cellular fragments) in the systemic circulation [114] and remote end-organ damage via PRR activation of distal inflammatory responses.

How such mechanisms are involved in the context of different pathogens, host-genomics, and concurrent therapeutic applications remains to be defined.

Despite the limitations of individual studies, the available taxonomic profiles for the human LRT in ARDS have informed the proposed ecological model (Fig. 11.1) with valuable empirical data. Patients with pneumonia-induced ARDS have

collapsed lung communities dominated by the causative pathogen [73–75], micro-aspiration from the URT is a major shaping force of LRT communities [18, 72, 74], and microbiota translocation across the gut-lung axis is a plausible explanation for the observed enrichment of gut-associated bacteria in many patients with ARDS [19, 76, 77]. Animal studies have offered novel insights on the impact of the altered lung microenvironment, including the effects of hyperoxia [100], antibiotics [77, 103], and bacterial toxins [77, 91, 103] on lung microbiota. Given the heterogeneous nature of ARDS and the complex array of critical care interventions, we expect patient-level and time-dependent variability in the contributions of various ecological forces on LRT microbiota. Such variability can make microbiome profile predictions unreliable, underlying the need for direct LRT sampling for microbiome studies.

Unanswered Questions and Future Research

With the widely shared goal of improving patient-centered outcomes in ARDS, microbiome-based investigations have to address key priorities and challenges in ARDS care [115]. *How can we prevent the development of ARDS? How can we save more lives among those who develop this devastating syndrome? How can we improve long-term functional outcomes in survivors?* ARDS results from a heterogeneous assemblage of different processes and manifests in various clinical presentations, and thus, the central premise for personalized care requires deeper understanding of the sources of clinical and biological heterogeneity [116].

Given that a LRT infection is often involved in the etiology of ARDS or complicates its course, culture-independent profiling of lung microbiota can have immediate implications for diagnosis and guidance of antimicrobial therapies. Rapid metagenomic sequencing performed directly in respiratory specimens (and not in isolated organisms from cultures) has been shown to be feasible and clinically valid, under different sampling, processing, and analytical protocols [54, 73, 117–121]. Although the clinical utility of metagenomics remains to be demonstrated by prospective investigations [120], the projected improvements in accuracy and timeliness of pneumonia diagnosis have tremendous implications for tailoring antimicrobials for individual patients and for strengthening antibiotic stewardship efforts in healthcare systems.

Linking microbiome profiles with host responses offers further opportunity for precision medicine approaches in ARDS. Langelier et al. have provided proof-of-concept evidence that simultaneous meta-transcriptomics of host and microbial RNA from the same respiratory specimen can help classify patients into those with or without a LRT infection [119]. Our group has recently shown that a simple index of dysbiosis (based on abundance of oral-origin bacteria and the alpha diversity of microbial community) is associated with the adverse hyperinflammatory subphenotype of systemic host-response [18], which may derive more benefit from immunosuppressive therapies such as glucocorticoids [122]. Such integrative approaches that tie microbiota and host immune responses can be harvested for identifying

which subsets of patients (and at what time points) may benefit from microbiome-targeted and/or immunomodulatory interventions.

Overall, the available evidence highlights the microbiome as a probable source of ARDS heterogeneity and a potentially modifiable risk factor to outcomes. With the proposed "expanded model of ARDS pathogenesis" (Fig. 11.2), we ought to consider the study of microbiota for mechanistic, diagnostic, predictive, and therapeutic implications in ARDS. In this context, we propose the following research questions as a broad scope set of 10 priorities for microbiome study in ARDS:

1. What are the mechanisms involved in host-microbiota interactions in the alveolar and gut mucosal epithelial interfaces that lead to innate immune activation and injury [123]? Can we prevent ARDS or reduce its severity by intervening in such pathways?
2. How are microbiota impacted by our standard practices and investigational interventions in ARDS care (e.g., airway hyperoxia, lung-protective ventilation, chlorhexidine mouth rinses, empiric antibiotics, etc.) [20, 21]? Is there preventable iatrogenic harm that we have not been considering and measuring?
3. Can culture-independent microbial sequencing technologies help us better diagnose infections in ARDS [117, 118, 120, 124]? Can microbiome studies help us improve antibiotic targeting and mitigate the antibiotic resistance crisis?
4. Can we use microbial warfare (e.g., bacteriocins or bacteriophages) as novel therapeutic options for VAP complicating ARDS [125]?
5. Can microbiome profiles help us distinguish responders from non-responders to specific interventions (i.e., predictive enrichment) [126]?
6. Can we safely and effectively modulate microbial communities in the lungs and gut of patients with ARDS to reverse harmful dysbiosis, eradicate pathogen colonization, or promote the survival of beneficial commensal organisms?
7. Is ICU dysbiosis preventable? How can we promote host-microbe homeostasis in the face of critical illness?
8. Once dysbiosis is established, what are the critical elements of commensal community homeostasis that are lost? How can we replace/restore the lost function even if we do not restore community structure?
9. Beyond bacteria, what is the role of proliferating fungi or viruses in the LRT during ARDS [127]?
10. Can we detect gut translocation in patients with ARDS? Is it harmful? Is it preventable?

In summary, the microbiome represents a new frontier in ARDS research. Lung and gut microbiota are emerging as important, modifiable contributors to the outcome of critical illness. Across many disciplines, personalized medicine is being redefined to include the inseparable microbial side of the human body [128]. With substantial clinical and experimental evidence supporting the role of microbiota in ARDS pathogenesis and outcome, the field is ripe for mechanistic and interventional studies that can translate discovery into measured benefits in clinical care of ARDS.

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The Future: Knowledge Gaps and Priorities

Stavros Garantziotis and Yvonne J. Huang

These are exciting times for lung microbiome research. As the chapters of this book have made evident, there is now a substantial body of literature supporting a central role for the lung microbiome in the maintenance of lung homeostasis and the development of lung disease. As we take stock of the significant new insights that we have gained in the past few years, we need to also consider persistent knowledge gaps and opportunities for further research that will translate these insights into tangible human health gains.

First, the majority of existing research into the lung microbiome has addressed bacterial species. However, we now know that other elements of the microbiome can play a role in host biology – viruses and fungi are perhaps less appreciated but are no less an important part of the microbiome meriting more attention. Second, host-microbiome interactions will need to be more closely scrutinized. It is no longer sufficient to characterize associations of microbiome with outcomes. Future research also will need to become more mechanistic and demonstrate the ways in which the microbiome interacts with the host immune system. This will enable us to establish basic principles that transcend organisms. It is well established that murine microbiomes differ between institutions [1], and we may expect regional variability in humans as well [2]. It is therefore perhaps unrealistic to expect

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experimental replication on the species or genus level between research groups. It seems unlikely that a specific genus solely promotes a chronic disease process, but rather that changes in ecological interactions among microbiota and with the host contribute to activation of specific immune pathways.

Host-microbiome interactions may run deeper than we think. Recent research has suggested that the mammalian host may co-opt signaling pathways from retroviruses in order to modulate its response to the microbiome [3]. Furthermore, bacteriophages may interact with both host and bacterial elements and influence the clearance of infections [4, 5]. Such (host × microbiome)-by-(microbiome × microbiome) interactions demonstrate the inherent complexity of cis- and trans- effects that will need to be elucidated in the future. This also highlights the analytical challenge that faces us, as we progress deeper into this field. Microbiome research is team science *par excellence*. Beyond the prerequisite experimental planning and sampling expertise, it requires specialized processing and measurement techniques (due to low lung microbiome biomass), innovative biostatistical analyses, as well as chemistry, metabolomic, and genetic skillsets. It is therefore probable that future microbiome research will cluster around institutions or groups that feature a high Shannon Index of infrastructure and skills.

All this is not to say that further observational data do not have immense value. On the contrary, we still do not understand a lot about the lung microbiome. Most studies are cross-sectional and based in a few locations. We do not understand whether, or to what extent, the respiratory microbiome is affected by environmental exposures: is environmental microbiome transferred to the airways [6]? If so, is it directly or via a relay station in the oropharynx? How do environmental exposures (e.g., pollution) modulate the microbiome? Is the airway microbiome the same in residents of North Carolina in the (hot, humid) summer and Michigan in the (cold, dry) winter? These are merely examples of the still outstanding questions that need observational data to answer them.

In summary, this book has exemplified the vast progress that has been accomplished in respiratory microbiome research and also highlighted the broad opportunities for future research that can be realized in the coming years. We hope that this book will provide the impetus for further research and entice researchers from across complementary fields to push forward this momentum.

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